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DISTRIBUTION AND MOLECULAR ANALYSIS OF AVIAN MALARIA VECTORS ON FOUR CENTRAL VIRGINIA PROTHONOTARY WARBLER (PROTONOTARIA CITREA) BREEDING SITES

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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LIST OF ABBREVIATIONS

An.	*Anopheles*
ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
CDC	Center for Disease Control
Cx.	*Culex*
DNA	Deoxyribonucleic Acid
GIS	Geographic Information System
P. citrea	*Protonotaria citrea*
PCR	Polymerase Chain Reaction
SPSS	Statistical Package for the Social Sciences
TAE	Tris-acetate-EDTA
WNV	West Nile virus
VCU	Virginia Commonwealth University

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ABSTRACT

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Avian malaria is a devastating disease that has decimated numerous bird species. This study sought to identify the vectors of avian malaria at four central Virginia Prothonotary warbler breeding sites. Twenty one thousand mosquitoes were collected and *Culex salinarius, Cx. erraticus,* and *Cx. pipiens/restuans* were found to be the dominant species at these sites. Geographic factors, such as crop land and forest type, were determined to be potential indicators

for species abundance variation between sites. Of the mosquitoes collected, ninety one (0.4%) were identified as blood fed. The blood fed mosquitoes were found to have fed on avian, mammalian, amphibian, and reptilian hosts and a 12.1% *Plasmodium* infection rate. Of the non-blood fed mosquito pools tested, Deep Bottom had the highest rate of infection (10.5%). Of the species tested, *Cx. salinarius, Cx. erraticus*, and *Cx. pipiens/restuans* were determined to be the most probable vectors of avian malaria the four sites.

CHAPTER I: DISTRIBUTION OF MOSQUITO VECTORS THROUGHOUT FOUR PROTONOTARIA CITREA BREEDING SITES IN CENTRAL VIRGINIA

INTRODUCTION

Prothonotary Warblers

The Prothonotary warbler, *Protonotaria citrea*, is a migratory species that breeds in central Virginia during the late spring and summer months, from late April through late July. The species overwinters in Central America and northern South America, and breed across the central and eastern United States, including Virginia (Cornell Lab of Ornithology). They are known to nest in small cavities in forested swamps and wetlands, and readily use artificial nest boxes (Blem, 1999). Their numbers have also been slowly declining over time (Blem, 1999). This species reproduction and nesting habits have been studied extensively using next boxes in central Virginia (Blem, 1999). At Dutch Gap Conservation Area, Deep Bottom Recreational Area, and Presquile National Wildlife Refuge, bird boxes have been established. In the summers of 2007 and 2008, the three breeding sites were found to have high levels of avian malarial infection (Grillo, Master's thesis). In a survey of 171 Prothonotary warblers, 114 (66.7%) were found to show evidence of avian malaria infections (Grillo, Master's thesis). Such a high level of infection demonstrates the need for determining how the disease transmitted.

Avian Malaria

Avian malaria is caused by apicomplexan parasites of the *Plasmodium* genus capable of infecting numerous bird species (Beadell *et al.* 2006; Belo *et al.*, 2009). Infection caused by *Plasmodium* has been epidemic in numerous tropical bird populations throughout the world (Foster *et al*, 2007; Belo *et al.*, 2009). Such epidemics occur in both wild and captive populations, leading to potentially harmful conditions for other organisms (Belo *et al.*, 2009). While avian malaria is not transmissible to humans, it is a major concern for bird populations and it could have devastating effects on wildlife if it were to become a major epidemic. With this in mind, it is important to understand the transmission of avian malaria in Virginia.

Role of Migratory Birds in the Spread of Disease Transmission

The role of migratory birds in the spread of diseases is an important consideration in predicting how infections will spread to human populations. Long distance migratory species are exposed more heavily to disease on their winter grounds, carrying a potentially high amount of infection to their summer breeding grounds (Lopez *et al.*, 2008). Large birds, such as corvids and owls, are known to amplify and transmit diseases, including those that can spread to humans (Gancz *et al.*, 2002; Fitzgerald *et al.*, 2003; Reisen *et al.*, 2006). Corvids are considered to be necessary to amplify the rate of West Nile virus (WNV) transmission, spreading it in many areas of the world (Reisen *et al.*, 2006). The role of smaller bird species in the spread of wildlife disease is not as well understood, though some research has been done, as in the case of cliff swallows (Brown *et al.*, 2007). Birds such as mourning doves (*Zenaida macroura*), eared doves (*Zenaida auriculata*), house finches (*Carpodacus mexicanus*), black-throated blue warblers (*Dendroica caerulescens*), and many other species of passerine have been found to be competent hosts for numerous bird diseases, such as avian malaria, avian influenza, WNV, and St. Louis

encephalitis (Komar, 2003; Mahmood, 2004; Fallon, 2006; Wood, 2007; Diaz, 2008; Reisen, 2009). It was determined that there was a relationship between migratory bird movement and the prevalence of a local infection, known as the Buggy Creek Virus (Brown *et al.*, 2007). In another study of migratory birds, differences in lineages of avian malaria were examined among black-throated blue warblers in North America (Fallon *et al.*, 2006). It was determined that while the parasite was present in different populations, the lineage of the parasite was not specified or grouped based upon breeding population or geographical region (Fallon *et al.*, 2006). There is also the concern of the birds mixing lineages of parasites, and potentially even gene recombination, occurring at bird wintering grounds. Fallon *et al.* (2006) suggested that the wintering grounds of the black-throated blue warbler would allow for increased transmission as well as the mixing of lineages within a single host. Koehler *et al.* (2008) determined that among wild Alaskan bird populations, the wide distribution and migratory patterns of the birds would allow for increased transmission and the reassortment of genes between avian influenza lineages. The authors further suspected that certain species of birds would be more likely to increase this transmission, and due to the diversity of species that migrate through that area, it is important to understand the role of different species of birds (Koehler *et al.*, 2008). Thus, there is a need for evaluating how small birds, both migratory and non-migratory, spread disease in a location.

Vectors of Avian Malaria

Mosquitoes are known vectors for avian malaria, transmitting the parasite between birds. Members of the *Anopheles* and *Culex* genera are known to be competent vectors for avian malaria (Burkot, 1984; Ejiri, 2008; Kent, 2009). *Culex* species are also known to frequently bite birds, allowing for these mosquitoes to transmit diseases between birds (Mackay *et al.*, 2010; Sawabe *et al.*, 2010). Ejiri *et al.* (2008) suggested that in order to determine the risk for infection

and to estimate the prevalence, it will be necessary to determine the positive rate of infection among known vectors.

Geographical Analysis

The prevalence of a disease can vary substantially due to differences in the geography and environmental conditions, such as proximity to the woodland edge, water, and differences in altitude (Wood *et al*. 2007). Fallon *et al.* (2006) also suggested that selective pressures may be different on migratory species than on non-migratory species due to the differences in location and geographical features. It has also been determined that the prevalence of disease spread by arthropods is influenced by the range of the vector species (Ejiri *et al.*, 2008). Numerous studies have associated abiotic environmental conditions and geographic features with vector mosquito species and distributions (Zhong *et al.*, 2003; Mercer *et al.*, 2005; Schafer *et al.*, 2006; Reiter *et al.*, 2007; Beketov *et al.*, 2010; Juliao *et al.*, 2010). Consequently both abiotic and geographic constraints on vector mosquito species distributions will also affect the prevalence of disease in bird populations. These constraints will also affect the prevalence and transmission of the disease in different bird populations, though mosquitoes were not considered a factor by Wood *et al.* (2007). In the same study, it was determined that proximity to water and woodland edge were significant geographical factors related to infection prevalence, and site altitude was also a significant indicator (Wood *et al.*, 2007). Temperature and precipitation have also been found to affect the transmission of WNV in the Chicago area, contributing to differences between lineages in populations of birds (Loss *et al.*, 2008). Distance to water was considered to factor into the wetness of the area, which in turn would affect the amount of suitable mosquito larval habitat, but this was not tested (Wood *et al.*, 2007). In more specific mosquito studies, various factors have been found to influence mosquito abundance. Wetlands, and the type of wetlands, as well

as numerous aspects of water habitat have been found to contribute to differences in abundance (Mercer *et al.*, 2005). Seasons, temperature, and vegetation changes have been found to be influential factors that affect mosquito abundance (Zhong *et al.*, 2003). The land use and forest cover of an area are important factors as well as the level of human influence (Schafer *et al.*, 2006; Zhong *et al.*, 2003).

Objective I

Due to the aforementioned information, it is important to identify the vectors present in the central Virginia breeding sites of Prothonotary warblers. The species and abundances of mosquitoes as well as the variation in both diversity and abundance of mosquitoes within each location are important factors to consider when attempting to understand the transmission cycle of avian malaria in Prothonotary warblers on four breeding sties in central Virginia. To achieve the objective of identifying potential vectors, the abundance and distribution of mosquito species will be compared on four Prothonotary warbler breeding sites in central Virginia.

Objective II

In addition, the differences between the breeding locations based upon habitat characteristics will be determined using Geographic Information Systems (GIS). The differences between the trap sites will be determined using multiple data layers and analyzed for ideal mosquito habitat. The results will be used to visually and quantitatively determine differences between trap locations.

MATERIALS AND METHODS

Study Sites and Collection

Mosquitoes were collected from four central Virginia locations. These locations were Dutch Gap Conservation Area of Chesterfield county, the Virginia Commonwealth University

Rice Center and Presquile National Wildlife Refuge of Charles City county, and Deep Bottom Recreational Area of Henrico county (Figure 1). At each location, two CO_2 baited (dry ice) Center for Disease Control (CDC) light traps (John W. Hock Company) and two gravid trap baited with organically rich water made up of pond water, local vegetation, and fish fertilizer (John W. Hock Company) were set near the edge of the water at each site. These traps were set in the early to mid afternoon, and picked up the following morning using the route shown in Figure 1. A total of thirteen trips were taken from June to August 2009. Insect collections were then placed in Petri dishes and frozen at 4˚C.

Identification and Pooling

All mosquitoes were sorted out from other insects and arachnids. The mosquitoes were then identified to genus and species levels using *A Key to the Mosquitoes of North Carolina and the Mid-Atlantic States* (Slaff, Apperson, and Rogers) provided by Henrico County Standing Water Initiative. In the case where species could not be determined, the genus was specified and the species designated as unknown, except in the case of *Cx. pipiens* and *Cx. restuans*. These two closely related species are morphologically indistinguishable and are often combined in many studies (White, 2006; Williams, 2007). Those that could not be morphologically identified were recorded as unknown and not used in subsequent molecular analysis. Mosquitoes were then pooled in groups of 10-50 individuals by date, location, genus and species. Any samples with fewer than ten mosquitoes were recorded, but not pooled for molecular testing. No differentiation was made between gravid and non-gravid mosquitoes for pooling. Blood fed mosquitoes, those that had taken a blood meal soon before capture, were visually identified and separated into single mosquito pools for molecular testing (Chapter 2).

Distribution Analyses

Geographic Information System (GIS) analyses were performed using ESRI ArcGIS 10.0. Using the most recent imagery available from the GIS server, a satellite image of the state of Virginia was used to create a point shapefile that contained all 8 specific trap locations, 2 at each Prothonotary warbler breeding site. At each of the four Prothonotary warbler breeding sites, a point was chosen equidistant from the two trap locations using a distance measuring tool. GPS coordinates were not used since they were not collected. Buffers were then generated around each location at 0.5, 1, and 2 miles. This was done due to the known flight capability and potential for dispersal among mosquito species (Schafer *et al.*, 2006). Various data layers for the state of Virginia were used for isolating information at the specific trap locations. A raster file, that divided the land into classifications of water, forest, and non-forest was used to determine the amount of forest cover in 30 m x 30 m pixels (vf_cm05_level1 from http://gisweb.deq.virginia.gov). A more specific land use raster file was used that categorized the land in the area into 14 different types (vf_cm05_level2 from http://gisweb.deq.virginia.gov). A file was used for different wetland types (20100122_VA_wetlands from http://www.fws.gov/wetlands/Data/DataDownload.html). Finally, a file containing the impaired, or slow moving or obstructed, rivers in the state was used (2008_Integrated_WQ_Report_Impaired_Rivers.zip from http://gisweb.deq.virginia.gov/). Following completion of the intersections and extractions with each file within the buffers, the buffer of 1 mile was chosen as the area for analysis of all the trap locations.

Statistical Analyses

Due to the few sites being examined, statistical analyses between the sites could not be conducted. Only four sites were examined here, and that did not provide sufficient degrees of freedom and power to perform statistical analyses.

Instead, a model was constructed using data from Henrico Standing Water Initiative in combination with the four sites tested here. Using the trap locations and mosquito collections at each of the 105 Henrico trap sites plus the four Prothonotary warbler breeding sites, it was attempted to determine what factors would contribute to higher or lower collection rates of three *Culex* species. Following the previously described GIS methods, data was extracted within a one mile buffer around each of the 105 collection sites. Simple linear regression was performed on each factor from the extracted GIS data to first determine which of the factors was significantly contributing to the increase or decrease of mosquito abundance. A second set of testing was done using a repeated measures model that determined which GIS factors are acting together to affect the abundance of the collected *Culex* mosquitoes. The factors determined would have either a positive or negative effect, based on the estimate value. In the presence of a negative estimate value, as the factor increased, the abundance would decrease. In turn, a positive estimate value would indicate an increase in abundance when that factor increased.

RESULTS

Species Abundance in Prothonotary Warbler Breeding Sites in Central Virginia

A total of 21,564 mosquitoes were collected over the course of 13 trap nights (Table 1). Of those, 91 (0.4%) were identified as blood-fed mosquitoes. A total of 18 species were collected (Table 2). The total abundance of each species collected is listed in Table 2. The species found were as follows: *Aedes albopictus, Aedes japonicus, Aedes triseriatus, Aedes*

vexans, Anopheles crucians, Anopheles punctipennis, Anopheles quadrimaculatus, Coquillettidia perturbans, Culex erraticus, Culex pipiens/restuans, Culex quinquefasciatus, Culex salinarius, Culex territans, Orthopodomyia signifera, Psorophora columbiae, Psorophora ferox, and *Toxorhynchites rutilus septentrionalis,* and *Uranotaenia sapphirina*. At all sites, the most abundant species by proportion were *Cx. salinarius, Cx. erraticus*, and *Cx. pipiens/restuans*. *Culex salinarius* was found to be the most abundant species, especially at the VCU Rice Center (Table 3). Using these three main species, the average abundance of collection at each site was calculated (Figure 2). The average collection per night by CO_2 baited light trap was also much higher at the Rice Center for the three species (Figure 3). *Culex pipiens/restuans* was most commonly collected at Deep Bottom by the gravid trap (Figure 4). Of the collected non-blood fed mosquitoes, 68.7% (14,788) were *Cx. salinarius,* 23.4% *Cx. erraticus* (5,034), and 1.7% *Cx. pipiens/restuans* (378). For the blood fed mosquitoes, the species of interest were the same. *Cx. salinarius* made up 33% (30), *Cx. erraticus* 37.4% (34), and *Cx. pipiens* 9.9% (9) of the collected mosquitoes. The variation of collection by week of the three predominant species by site can be found in Figures 5-7.

Description of Sites using Resulting Geographic Features

The results of GIS extraction allowed for the four sites to be compared both visually and quantitatively. Dutch Gap was the only site that appeared different using the forest cover data (Figure 8). There was noticeably more non-forest area. When the area was calculated in meters squared, there were apparent differences between the sites based on the type of forest cover that was present (Table 4). Dutch Gap had a much higher amount of non-forest areas, while the Rice Center had more water and Deep Bottom had the most forested area (Table 4). When the land was divided between 14 categories, the area of each category in meters squared varied greatly,

though it was more difficult to visually determine differences on the generated map (Table 5; Figure 9). At Dutch Gap, crop land, hardwood forest, and water are the highest, and there are also high levels of residential land use type. It was also the only sight of those tested to have rooftop and natural barren areas. This indicates a higher amount of human interference at this location, and lower amounts of ideal mosquito breeding grounds. The VCU Rice Center has mostly hardwood forest, crop land, and water areas. It also had the lowest amount of mixed forest. Presquile has high area in pine forest, hardwood forest, and crop land, and the lowest amount of residential areas and pavement. Deep Bottom had a high area of hardwood forest, water, and crop land, and the highest area of forest harvest, bare soil, and salt marsh. The area around Deep Bottom appears to have the greatest area of impaired river in feet while Presquile has the second highest (Figure 10; Table 6). The wetlands around each also showed some variance (Table 7; Figure 11). At Dutch Gap, Presquile, and Deep Bottom, there are mostly riverine and freshwater forested/shrub wetlands. The Rice Center has mostly riverine and lake type wetlands. In summary, Dutch Gap was determined to be an area with a great amount of human activity and influence, with hardwood forests, cropland, riverine, and freshwater forested shrubs present. The VCU Rice Center was found to have a great amount of riverine and lake areas, as well as hardwood forests and cropland. The Presquile location had high amounts of pine forests, hardwood forests, crop land, riverine and freshwater forest/shrub wetlands. Deep Bottom was the only site with a high amount of impaired rivers in addition to riverine and freshwater forest/shrub wetlands and hardwood forests and crop land.

Statistical Results

In the results of the simple linear regressions analyzing, a number of factors were found to significantly affect the abundance of the three predominant *Cx.* species (Tables 8-10). For *Cx,*

salinarius, it was determined that numerous factors were found to be significant, including water and forest cover, residential/industrial areas, pavement, hardwood and pine forest, crop, bare soil, salt marsh, freshwater emergent wetland, lake, and riverine areas (Table 8). In the repeated measures model, it was determined that mine and quarry, hardwood forest, forest harvest, and crop land areas were significant as predictors for the abundance of *Cx. salinarius* (Table8). *Culex erraticus* was found to be significantly influenced by water and forest cover, pavement, residential/industrial, hardwood, pine, and mixed forest, crop land, salt marsh, freshwater emergent wetlands, freshwater pond, lake and riverine areas (Table 9). When considered together using the repeated measures model, water, pavement, mine and quarry, and salt marsh areas were found to be significant predictors together to affect *Cx. erraticus* abundance (Table 9). The simple linear regression result for *Culex pipiens/restuans* found that forest cover, pavement, residential/industrial, hardwood, pine, and mixed forest, forest harvest, and impaired rivers (Table 10). The model produced water, mine and quarry, pine and mixed forest, forest harvest, crop land, and bare soil as significant affecters for the abundance of *Cx. pipiens/restuans* (Table 10). Based on these results, the Rice Center is the most suitable location for *Cx. salinarius*, followed by Presquile and Deep Bottom, with Dutch Gap the least suitable due to the amount of residential and industrial areas. *Cx. erraticus* abundance can be projected to be high at the Rice Center and Deep Bottom, with Presquile and Dutch Gap providing less adequate habitat areas for this species. The abundance of *Cx. pipiens/restuans* can be projected to be most abundantly found at Dutch Gap, with the other three sites being less suitable due to the lack of residential/industrial areas.

DISCUSSION

There were a total of 18 species of mosquito found during the collection period of June 15 to August 10, 2009. There are 51 species within the state of Virginia (Henrico Standing Water Initiative, personal communication), and due to the high number of species found, those mosquitoes that were collected were determined to be highly representative of the areas in which they were collected. There was concern that this would not be the case, as the traps that were used do have some natural trap bias. The light traps will more readily collect those mosquitoes seeking hosts, while the gravid traps are more biased toward *Cx. pipiens/restuans* collection due to the design of the trap and the inclination of *Cx. pipiens/restuans* to search for organically rich water in which to lay eggs (Reisen *et al.*, 1999).

Due to the high numbers of *Cx. erraticus, Cx. pipiens/restuans*, and *Cx. salinarius*, subsequent GIS analyses focused on these three species. While the distributions of these three species by weekly collection can be seen in Figures 5-7, these distributions could not be analyzed due to the lack of degrees of freedom and resulting statistical power, as well as the lack of suitable GIS data, such as weather or precipitation, which could account for the changes in abundance seen at each site. It is also important to note that there was no distinction made in this study between *Cx. pipiens* and *Cx. restuans*. This was done due to the fact that the two species are difficult to identify morphologically and can only be conclusively identified by molecular analysis. Further, the abundance data was examined using the means per collection period to equalize the amount of effort put into the collection of each species at each location (Table 3, Figures 2-4).

While the sites did not appear to have a substantial level of difference in forest cover, land use, wetlands, or impaired rivers after GIS analyses, there were some relationships that may

explain the differences in mosquito species distribution. The Rice Center had a higher area of riverine wetlands, but not significantly so from the other sites. Dutch Gap had the highest amounts of human activity areas, while Presquile had a high amount of forested areas and Deep Bottom had a high amount of impaired rivers. These differences could be seen numerically as well as visually, but were there were not enough replicates of the sites to determine if the sites were statistically significant from one another. Further, the land use was designated to either forest cover or a more specific land use data set, as both forest cover and human interference are found to influence mosquito abundance (Reiter *et al.*, 2007; Schafer *et al.*, 2006; Mercer *et al.*, 2005). The wetlands were also determined around each site, as the extent and type of wetland has been found to influence the presence of mosquitoes (Mercer *et al.*, 2005). Different types of data layers were used with the objective of addressing the differences between the sites.

The Rice Center, since it had such a high number of *Cx. salinarius,* was the first to be examined. According to the GIS analyses, there are a high number of riverine areas at the Rice Center, but this does not appear different from Dutch Gap or Deep Bottom (Table 7). However in comparison to the other locations, the Rice Center has less freshwater wetlands (Table 7). It is probable that *Cx. salinarius* prefers areas with a low amount of freshwater wetlands. It is known that *Cx. salinarius* favors marsh areas and areas with a high level of grass organic matter (Fairfax County Health Department). It may be that this grass organic matter contributes a favorable environment for *Cx. salinarius*, as the Rice Center, upon visual inspection, seems very suitable according to this description. Using the impaired river data as an indicator for high levels of organic matter in the water or stagnant areas, the Rice Center had a fairly high amount of impaired river, but not high enough to be considered significant (Table 6).

Secondly, Deep Bottom was examined. It was the only location where the highest number of *Cx. pipiens/restuans,* a known bird biting mosquito, was collected. The first feature that distinguished Deep Bottom from the other three sites was the high amount of feet of impaired river that existed around the trap locations (Table 6). Using impaired rivers as an indicator for non-flowing and organic rich water, this area seems to be ideal for *Cx. pipiens/restuans* (Fairfax County Health Department). Furthermore, Deep Bottom had high area of riverine and freshwater forested/shrub wetlands. This indicates that while there are high levels of flowing water, there is almost an equal amount of freshwater forested/shrub wetlands (Table 7). So not only are there many water bodies that would be suitable, but there would also be a high amount of shrub and forest areas where the mosquitoes could also lay eggs or rest, as the species is known to prefer forested/shrub wetland habitats for breeding (Fairfax County Health Department). Of the surrounding land use types, there is a greater amount of crop land compared to the other sites (Table 5). This supports the hypothesis that the water in the area is more likely organically rich and preferable breeding areas for *Cx. pipiens/restuans*. There was a low area of freshwater pond, but the amount was still higher than either Presquile or the Rice Center (Table 6).

At the Presquile location, it is important to note that collection was not done on the actual Presquile Island, but across the river at a residential location. This may not be the most accurate representation of the mosquito populations on the island, but this was the only option given logistics and material restrictions of the study. With that caution, Presquile actually collected more *Cx. erraticus* than Dutch Gap (Table 3). This is somewhat unexpected as *Cx. erraticus* is known to favor pond margin areas (Fairfax County Health Department), and Dutch Gap has a higher area of freshwater pond wetland than Presquile (Table 6). Presquile had the highest area

of freshwater forested/shrub wetlands (Table 6). There was also a much higher area of forest cover (Table 4), and this was reflected in the fact that the location had the highest area of pine forest (Table 5). These features made it surprising that there were not greater differences between this site and the other three when it came to mosquito distribution.

Dutch Gap had the lowest diversity of mosquitoes (Table 3). It was the only site where *Orthopodomyia signifera* was collected (Table 3), which is a known bird biting mosquito that also favors wooded areas (United States Geological Survey, 2001). This was surprising since Dutch Gap actually had the lowest amount of forest cover (Table 4). Dutch Gap also had the highest level of human interference as inferred from having the highest area of pavement, rooftop, and residential land types (Table 5). For the wetland data, the only distinguishing feature of Dutch Gap is that it was the only location to have the "other" classification (Table 7).

In conclusion, there are species present at each site that are capable of spreading avian diseases, notably the three predominantly collected *Cx.* species. It is probable that multiple mosquito species at the four locations are acting as competent vectors for avian malaria. This could partially explain the high levels of avian malaria infection in birds observed in previous studies at these sites (Grillo, Master's thesis).

As shown in previous studies, the geographical features are very important to determine suitable mosquito habitats and potential areas for disease transmission (Wood *et al.*, 2008; Reiter *et al.*, 2007; Schafer *et al.*, 2006; Mercer *et al.*, 2005; Zhong *et al*., 2003). In this study, there were different geographical features at each site that were then tested to attempt to determine the suitability of each location for the three predominant mosquito species.

Using the simple linear regression results, it was determined that the abundance of *Cx. salinarius* will increase in the presence of water, hardwood and pine forest, crop land, bare soil,

freshwater emergent wetland, lake and riverine areas, as indicated by the estimate value (Table 8). The abundance will decrease in the presence of residential/industrial areas, pavement, and non-forest. These are not surprising, as increased human presence, as indicated by these types of areas, will cause a decrease in mosquito populations. Further, the freshwater emergent wetlands, lake and riverine areas support the increase in abundance due to the known characteristics for breeding habitats preferred by *Cx. salinarius*. According to the repeated measures model, mine and quarry, hardwood forest, and crop land will contribute to the increase of *Cx. salinarius*, while forest harvest will contribute to the decrease in abundance (Table 8). These results indicate an area similar to the Rice Center, and provide initial evidence of why the Rice Center has such a large abundance of *Cx. salinarius.*

In the case of *Cx. erraticus*, the presence of water, hardwood, pine, and mixed forest, crop land, salt marsh, freshwater emergent wetlands, freshwater pond, lake and riverine areas will increase the abundance (Table 9). The presence of non-forest areas, such as residential/industrial, will contribute to decreasing the abundance (Table 9). With the model, it was determined that water, mine and quarry, and salt marsh will contribute to the increase of the species together, while pavement will decrease the abundance (Table 9). The wetland types of area are suitable for *Cx. erraticus*, and this provides some evidence for habitat suitability for the species.

Culex pipiens/restuans had fewer factors that contributed significantly to affecting the abundance. According to the simple linear regressions, pavement and residential/industrial areas will increase the abundance of mosquitoes, while hardwood, pine, and mixed forest, forest harvest, and impaired rivers will decrease the abundance (Table 10). This was unexpected, as *Cx. pipiens/restuans* is known to favor organically rich water which could be indicated by the

amount of impaired rivers in the area, and this contradicts the previously offered explanation of why Deep Bottom collected the most *Cx. pipiens/restuans.* In the repeated measures model, the only factors that contributed to increasing the abundance were water, crop land, and mine and quarry (Table 10). The results for *Culex pipiens/restuans* were unclear and not conclusive, showing the need for further analysis to determine a suitable habitat for this species.

These statistical results demonstrate that there are distinct differences between the sites which can be used to determine which locations have a more suitable habitat for mosquito species. These analyses also provide some evidence for predicting which sites should be focused on as suitable locations for collecting avian malaria vectors and where avian malaria could be transmitted between migratory and non-migratory birds.

Future Studies

In the future, the four Prothonotary warbler breeding sites could be more comprehensively sampled. Only four traps were placed at each location, two of each type (CDC light trap and gravid trap), and this did not provide enough power for the study to use statistical analyses. The sampling period could start earlier in May and end August to better coincide with the period that Prothonotary warblers are present in Virginia. This would allow more comprehensive coverage of both the Prothonotary warbler breeding season as well as the mosquito breeding season. This was not done for this study due to various logistical restraints. For Presquile Island, it would be beneficial to determine the mosquito vector distribution on the actual island, where the mosquitoes are more likely to come into contact with the Prothonotary warblers tracked by VCU. Finally, other data layers could be used for GIS analyses to answer questions about the seasonal distributions of mosquitoes and to better determine geographic or abiotic features that influence mosquito species presence. Temperature data was not recorded at

the time of collection, and no suitable temperature or weather data could be found at each of the four sites for this analysis. In the future, it would be beneficial to determine seasonal and meteorological conditions that affect changes in mosquito distribution. The amount of rainfall would be especially beneficial, as would temperature, as both have been found to be important indicators for differences in mosquito distributions (Loss *et al.*, 2008; Beketov *et al.*, 2010; Juliao *et al.*, 2010). Even soil composition may be considered in the future, as would the different flora distributions at the breeding sites (Beketov *et al.*, 2010).

TABLES

Table 1. Dates of collection trips taken during the summer of 2009

Travel Dates	
Place Traps	*Pick Up Traps*
15-Jun	16-Jun
22-Jun	23-Jun
29-Jun	30-Jun
2-Jul	3-Jul
7-Jul	8-Jul
13-Jul	14-Jul
16-Jul	17-Jul
20-Jul	21-Jul
23-Jul	24-Jul
27-Jul	28-Jul
3-Aug	4-Aug
6-Aug	7-Aug
10-Aug	11-Aug

Table 2. List of mosquito species collected and total abundance

Species	Non-Blood Fed Mosquitoes					Blood Fed Mosquitoes				
	Rice Center	Presquile	Dutch Gap	Deep Bottom	Percentage	Rice Center	Presquile	Dutch Gap	Deep Bottom	Percentage
Aedes albopictus	44	13	26	34	0.5	1	1	1	0	3.3
Aedes japonicus	2	0	1	2	0.0	0	0	0	0	0.0
Aedes triseriatus	0	1	3	0	0.0	0	0	0	0	0.0
Aedes vexans	55	27	148	62	1.4	1	1	1	4	7.7
Anopheles crucians	184	2	17	194	1.8	0	0	0	0	0.0
Anopheles punctipennis	19	3	12	14	0.2	0	0	0	0	0.0
Anopheles quadrimaculatus	37	19	21	84	0.7	0	1	1	0	2.2
Coquillettidia perturbans	16	2	192	10	1.0	0	0	1	0	1.1
Culex erraticus	1811	952	532	1739	23.4	8	6	8	12	37.4
Culex pipiens/restuans	56	53	65	204	1.8	0	0	1	8	9.9
Culex quinquefasciatus	0	0	0	0	0.0	0	0	0	1	1.1
Culex salinarius	13397	444	211	736	68.7	26	0	1	3	33.0
Culex territans	1	0	0	0	0.0	0	0	0	0	0.0
Orthopodomyia signifera	0	0	1	0	0.0	0	0	0	0	0.0
Psorophora columbiae	11	3	0	0	0.1	0	0	0	0	0.0
Psorophora ferox	0	0	0	8	0.0	0	0	1	0	1.1
Toxorhynchites rutilus septentrionalis	2	0	0	0	0.0	0	0	0	0	0.0
Unknown	4	2	0	0	0.0	0	0	0	0	0.0
Unknown *Anopheles*	1	0	2	0	0.0	0	0	0	0	0.0
Unknown *Culex*	0	0	0	4	0.0	0	0	0	0	0.0
Unknown *Psorophora*	0	1	0	0	0.0	0	0	0	0	0.0
Uranotaenia sapphirina	4	9	1	27	0.2	2	0	1	0	3.3
Total	15644	1531	1232	3118	21525	38	9	16	28	91

Table 3. The total abundance of the mosquitoes collected by site

	Location			
Species	Dutch Gap	Rice Center	Presquile	Deep Bottom
Aedes albopictus	26	44	13	34
Aedes japonicus	1	2	0	2
Aedes triseriatus	3	0	1	0
Aedes vexans	149	56	28	66
Anopheles crucians	17	184	2	194
Anopheles punctipennis	12	19	3	14
Anopheles quadrimaculatus	22	37	20	84
Coquillettidia perturbans	193	16	2	10
Culex erraticus	540	1819	958	1751
Culex pipiens/restuans	66	56	53	212
Culex salinarius	212	13423	444	739
Culex territans	0	2	0	0
Orthopodomyia signifera	1	0	0	0
Psorophora columbiae	0	11	3	0
Psorophora ferox	1	0	0	8
Toxorhynchites rutilus septentrionalis	0	2	0	0
Unknown	0	4	2	0
Unknown *Anopheles*	2	1	0	0
Unknown *Culex*	0	0	0	4
Unknown *Psorophora*	0	0	1	0
Uranotaenia sapphirina	2	6	9	27

Table 4. The area in square meters of the forest cover data layer extracted within one mile of each trap location

		Location			
		Dutch Gap	Rice Center	Presquile	Deep Bottom
Type Designation	Water	806.6	882.8	394.4	378.0
	Forest	7165.3	11293.8	13970.8	11558.3
	Non-forest	4215.9	1804.6	2516.8	3809.8

Table 5. The area in square meters of the land use data layer extracted within one mile of each trap location

	Location			
Land Type	Dutch Gap	Rice Center	Presquile	Deep Bottom
Water	806.6	882.8	394.4	378.0
Pavement	70.2	15.4	6.3	22.0
Rooftop	0.4	0.0	0.0	0.0
Residential/Industrial	265.3	115.6	96.9	120.4
Natural Barren	0.1	0.0	0.0	0.0
Mine/Quarry	0.3	0.1	0.0	0.0
Hardwood Forest	3339.2	5529.7	5946.6	5465.6
Pine Forest	87.0	299.5	585.5	217.2
Mixed Forest	147.8	49.3	141.9	123.7
Forest Harvest	9.5	35.2	14.1	45.3
Grassland	0.0	0.1	0.1	0.0
Crop	571.4	506.5	918.5	1205.9
Bare Soil	39.9	6.8	1.8	43.4
Salt Marsh	4.6	0.8	10.5	10.6

Table 6. The feet of the impaired river data layer extracted within one mile of each trap location

	Location			
	Dutch Gap	Rice Center	Presquile	Deep Bottom
Feet	111.6	4228.1	8455.9	20262.1

Table 7. The acreage of the wetland data layer extracted within one mile of each trap location

	Location			
	Dutch Gap	Rice Center	Presquile	Deep Bottom
Freshwater Emergent Wetland	28	11	79	42
Freshwater Forested/Shrub Wetland	154	30	521	218
Freshwater Pond	28	14	13	25
Riverine	493	615	306	291
Lake	65	64	15	0
Other	4	0	0	0

Table 8. Simple linear regression and multivariable repeated measures model results for *Culex salinarius*

Individual Variables				
Variable	**Estimate**	**Std. Error**	**z value**	**Pr(>|z|)**
Water	0.469	0.0517	9.06	<2e-16
Forest	0.5596	0.1618	3.458	0.000543
Non-forest	-0.7199	0.1411	-5.101	3.38E-07
Water	0.5054	0.0556	9.079	<2e-16
Pavement	-0.5478	0.1621	-3.379	0.000727
Rooftop	0.1151	0.1751	0.657	0.511
Residential/Industrial	-0.7345	0.1426	-5.152	2.58E-07
Mine/Quarry	0.1652	0.1752	0.943	0.346
Hardwood Forest	0.7117	0.1412	5.039	4.69E-07
Pine Forest	0.3421	0.1719	1.99	0.0466
Mixed Forest	0.2014	0.18	1.119	0.263
Forest Harvest	0.0007	0.1852	0.004	0.997
Grassland	0.0207	0.1796	0.115	0.908
Crop	0.538	0.148	3.636	0.000277
Bare Soil	0.1436	0.174	0.825	0.409
Salt Marsh	0.4073	0.0668	6.091	1.12E-09
Freshwater Emergent Wetland	0.4562	0.1697	2.688	0.00718
Freshwater Pond	0.2788	0.1741	1.602	0.109
Lake	0.6216	0.0991	6.271	3.60E-10
Other	0.4029	0.1523	2.645	0.00816
Riverine	0.4593	0.0491	9.35	<2e-16
Impaired Rivers	-0.0549	0.1717	-0.32	0.749
Multiple Variables				
Variable	**Estimate**	**Std. Error**	**z value**	**Pr(>|z|)**
(Intercept)	-3.455563	0.2499718	-13.824	< 2e-16
Mine/Quarry	1.1752443	0.6859116	1.713	0.086638
Hardwood Forest	0.0006484	0.0001268	5.112	3.18E-07
Forest Harvest	-0.0134074	0.0037349	-3.59	0.000331
Crop	0.0018132	0.0007289	2.488	0.012858

Table 9. Simple linear regression and multivariable repeated measures model results for *Culex erraticus*

Individual Variables				
Variable	**Estimate**	**Std. Error**	**z value**	**Pr(>|z|)**
Water	0.5329	0.082	6.499	8.08E-11
Forest	1.0944	0.2179	5.022	5.10E-07
Non-forest	-1.1996	0.1892	-6.341	2.29E-10
Water	0.5796	0.0877	6.605	3.99E-11
Pavement	-1.3357	0.2199	-6.073	1.26E-09
Rooftop	0.3358	0.2437	1.378	0.168
Residential/Industrial	-1.1026	0.1989	-5.543	2.97E-08
Mine/Quarry	0.1292	0.2487	0.519	0.603
Hardwood Forest	1.1237	0.1931	5.818	5.95E-09
Pine Forest	0.7797	0.2357	3.308	0.00094
Mixed Forest	0.5961	0.2495	2.389	0.0169
Forest Harvest	0.4668	0.2535	1.842	0.0655
Grassland	0.091	0.251	0.363	0.717
Crop	1.0212	0.1952	5.231	1.69E-07
Bare Soil	0.386	0.2469	1.564	0.118
Salt Marsh	0.5886	0.0899	6.545	5.96E-11
Freshwater Emergent Wetlands	0.5918	0.2429	2.437	0.0148
Freshwater Pond	0.7118	0.2415	2.948	0.0032
Lake	0.6909	0.1496	4.618	3.88E-06
Other	0.548	0.2156	2.542	0.011
Riverine	0.51261	0.0794	6.453	1.10E-10
Impaired Rivers	0.04214	0.2447	0.172	0.863
Multiple Variables				
Variable	**Estimate**	**Std. Error**	**z value**	**Pr(>|z|)**
(Intercept)	-1.487666	0.442981	-3.358	0.000784
Water	0.004612	0.001626	2.836	0.004562
Pavement	-0.008545	0.001844	-4.634	3.59E-06
Mine/Quarry	1.508171	0.805233	1.873	0.061074
Salt Marsh	0.415018	0.125121	3.317	0.00091

Table 10. Simple linear regression and multivariable repeated measures model results for *Culex pipiens/restuans*

Individual Variables				
Variable	**Estimate**	**Std. Error**	**z value**	**Pr(>|z|)**
Water	0.08272	0.06272	1.319	0.187
Forest	-0.58796	0.13508	-4.353	1.35E-05
Non-forest	0.45508	0.12957	3.512	0.000444
Water	0.099	0.06724	1.473	0.141
Pavement	0.36186	0.13999	2.585	0.00974
Rooftop	0.036341	0.15147	0.24	0.81
Residential/Industrial	0.33234	0.13514	2.459	0.0139
Mine/Quarry	0.139706	0.15067	0.927	0.354
Hardwood Forest	-0.36009	0.13157	-2.737	0.0062
Pine Forest	-0.427788	0.14289	-2.994	0.00276
Mixed Forest	0.642434	0.14699	-4.37	1.24E-05
Forest Harvest	-0.703589	0.14744	-4.772	1.82E-06
Grassland	0.193634	0.14998	1.291	0.197
Crop	-0.15756	0.13482	-1.169	0.243
Bare Soil	-0.257527	0.14814	-1.738	0.0821
Salt Marsh	0.1141	0.06807	1.676	0.0937
Freshwater Emergent Wetlands	-0.109985	0.15032	-0.732	0.464
Freshwater Pond	-0.111749	0.15064	-0.742	0.458
Lake	0.11748	0.10249	1.146	0.252
Other	0.11269	0.13601	0.828	0.407
Riverine	0.09149	0.0605	1.512	0.13
Impaired Rivers	-0.35727	0.14236	-2.51	0.0121
Multiple Variables				
Variable	**Estimate**	**Std. Error**	**z value**	**Pr(>|z|)**
(Intercept)	0.7718318	0.2545794	3.032	0.00243
Water	-0.0105321	0.0054534	-1.931	0.05345
Water	0.0118353	0.0054103	2.188	0.0287
Mine/Quarry	1.3066657	0.6406221	2.04	0.04138
Pine Forest	-0.0013347	0.0007919	-1.686	0.09189
Mixed Forest	-0.0023583	0.0016218	-1.454	0.14591
Forest Harvest	-0.0113387	0.0036091	-3.142	0.00168
Crop	0.0015566	0.0007521	2.07	0.03849
Bare Soil	-0.0316505	0.011564	-2.737	0.0062

FIGURES

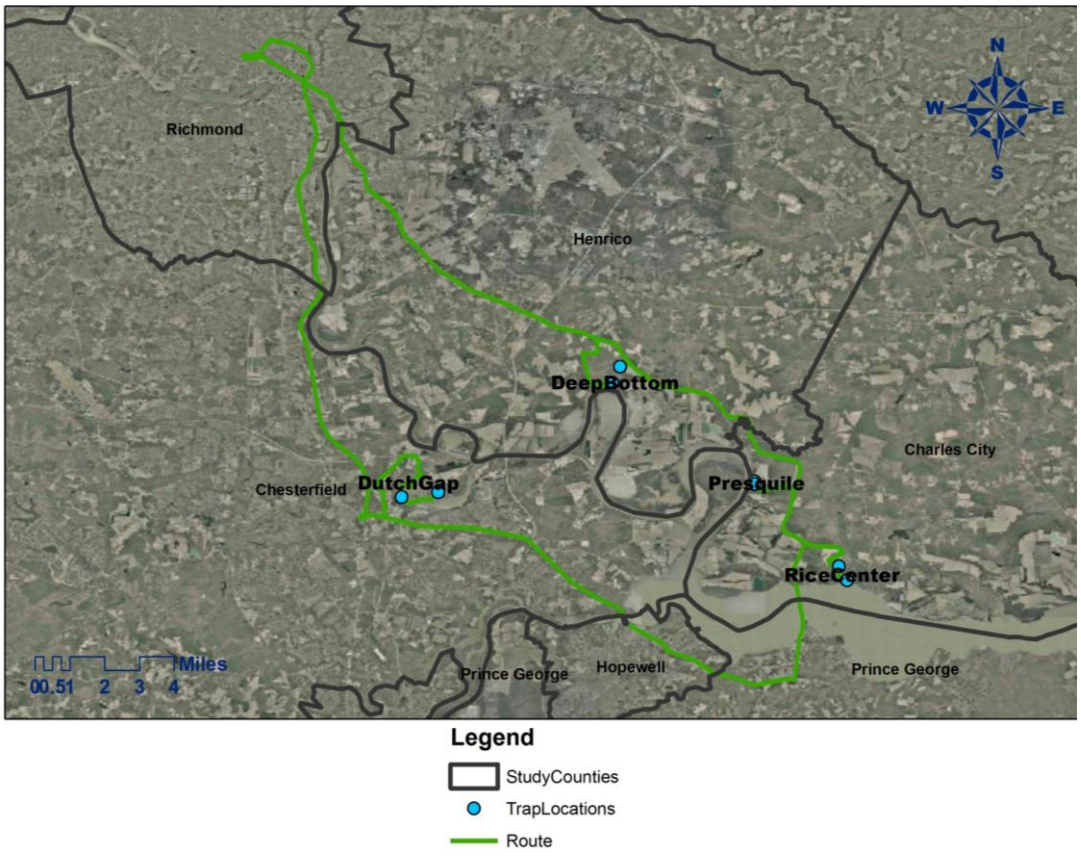

Figure 1. Trap location and route taken during mosquito trapping of summer 2009

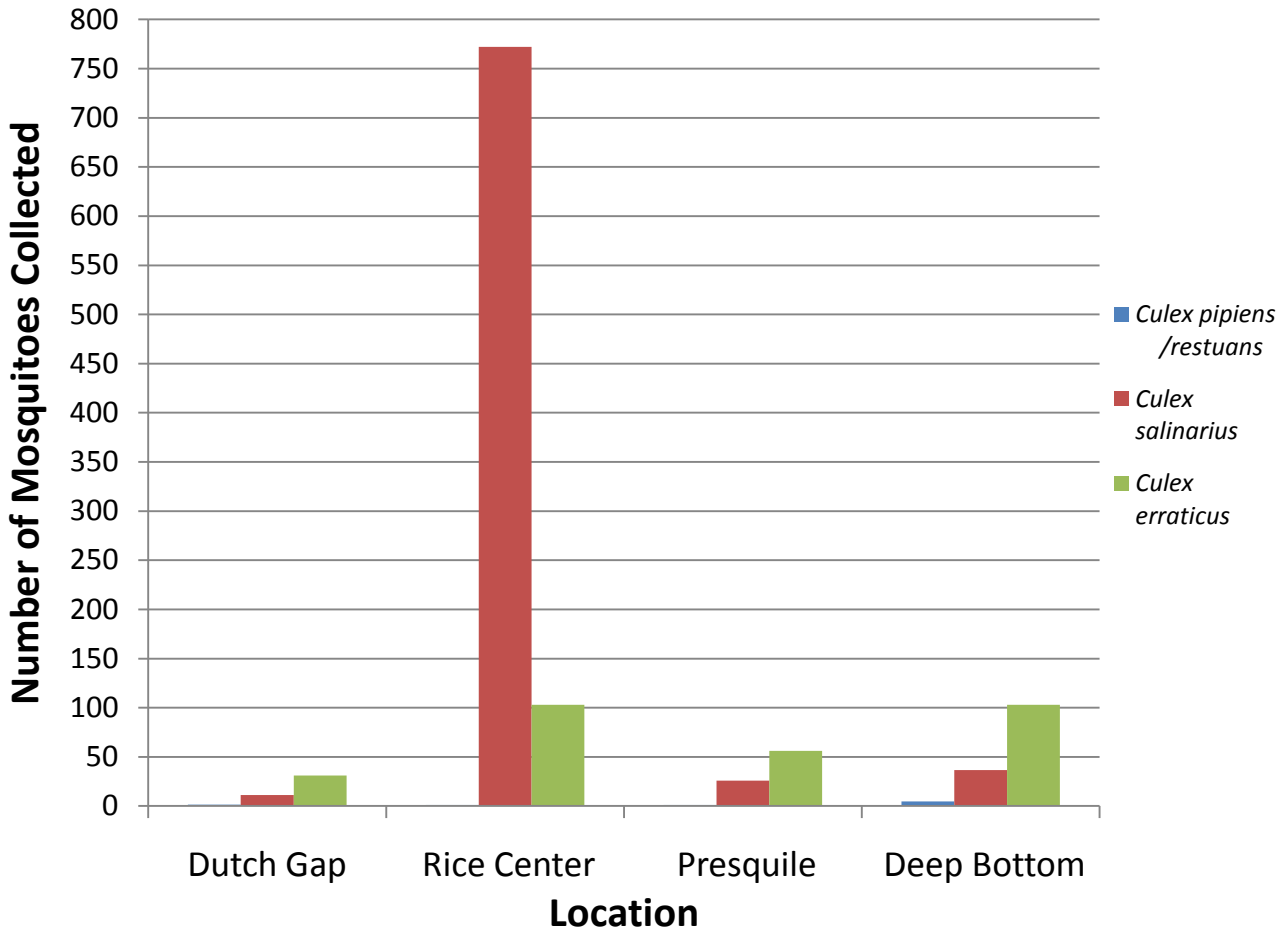

Figure 2. Total proportional abundance of mosquito species collected at each site

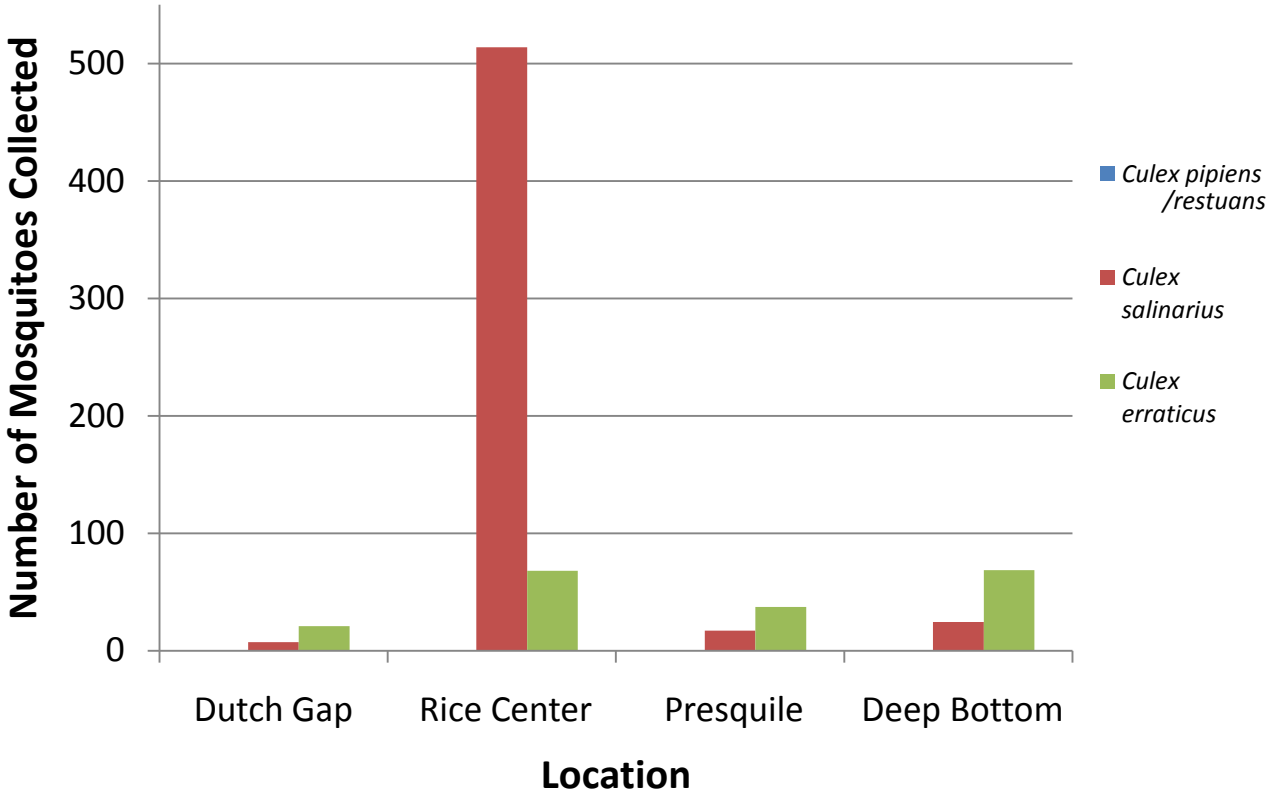

Figure 3. Proportional abundance of mosquito species collected by light trap at each site

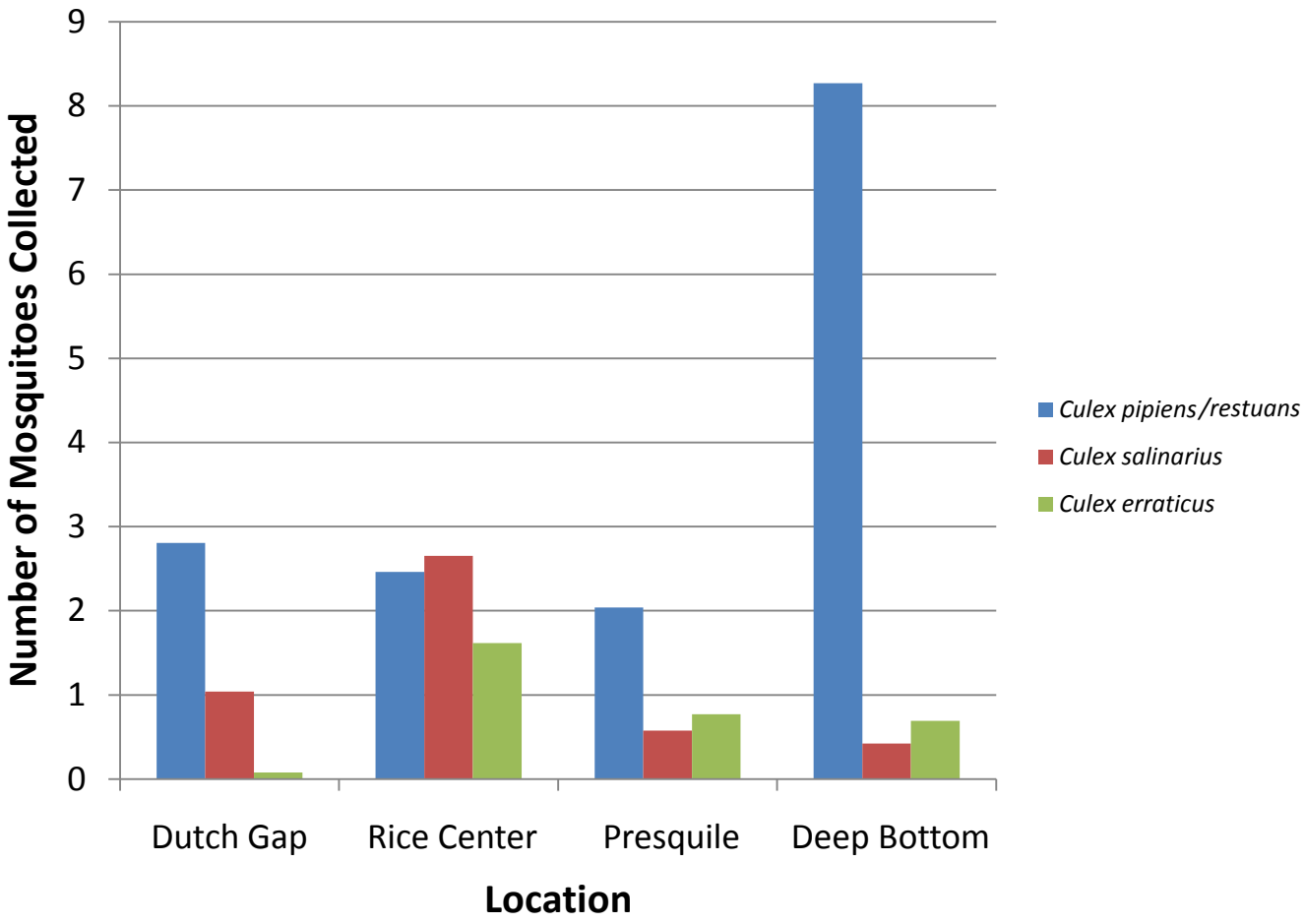

Figure 4. Proportional abundance of three mosquito species collected by gravid trap at each site

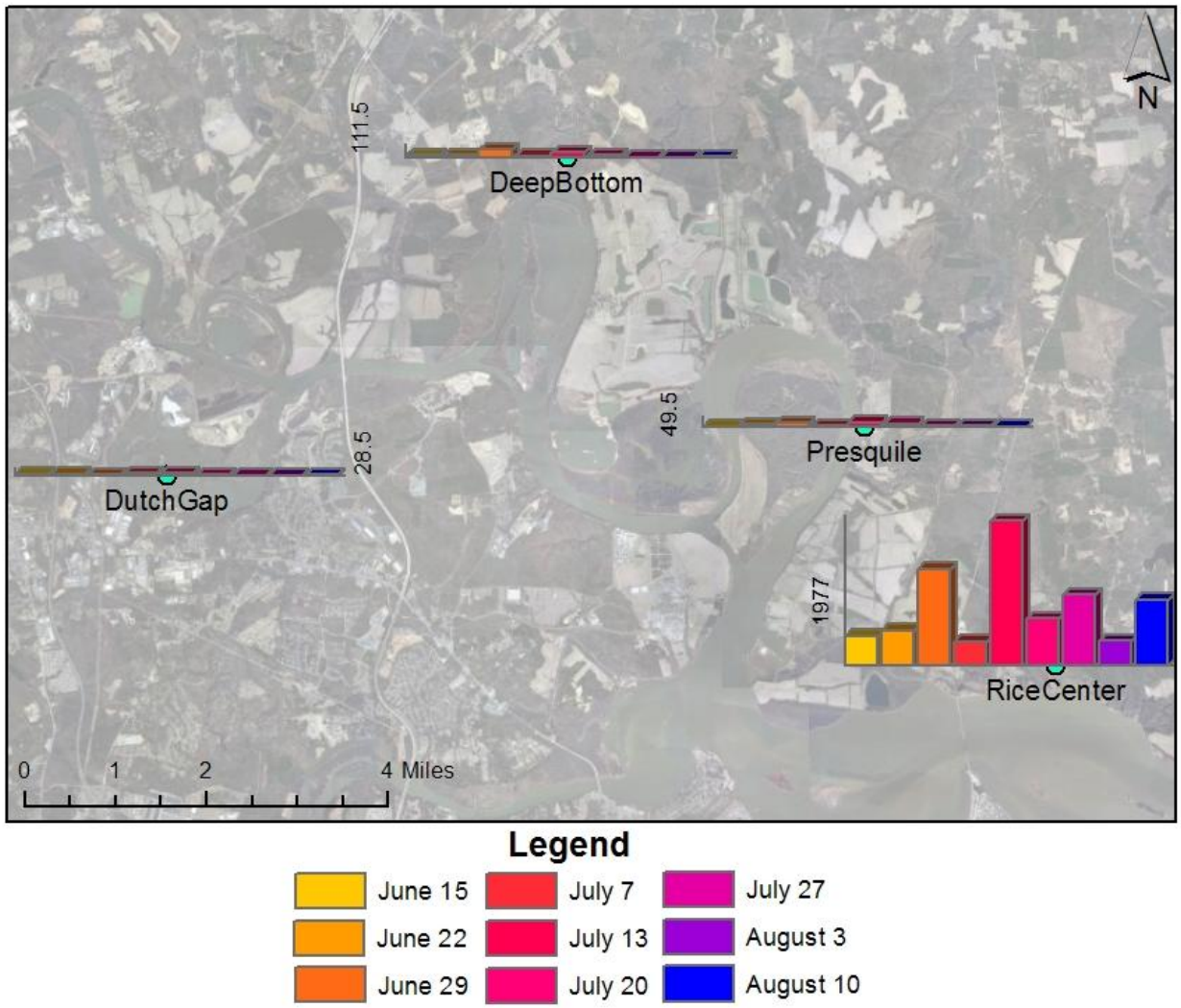

Figure 5. Distribution of *Culex salinarius* collected at each site per week

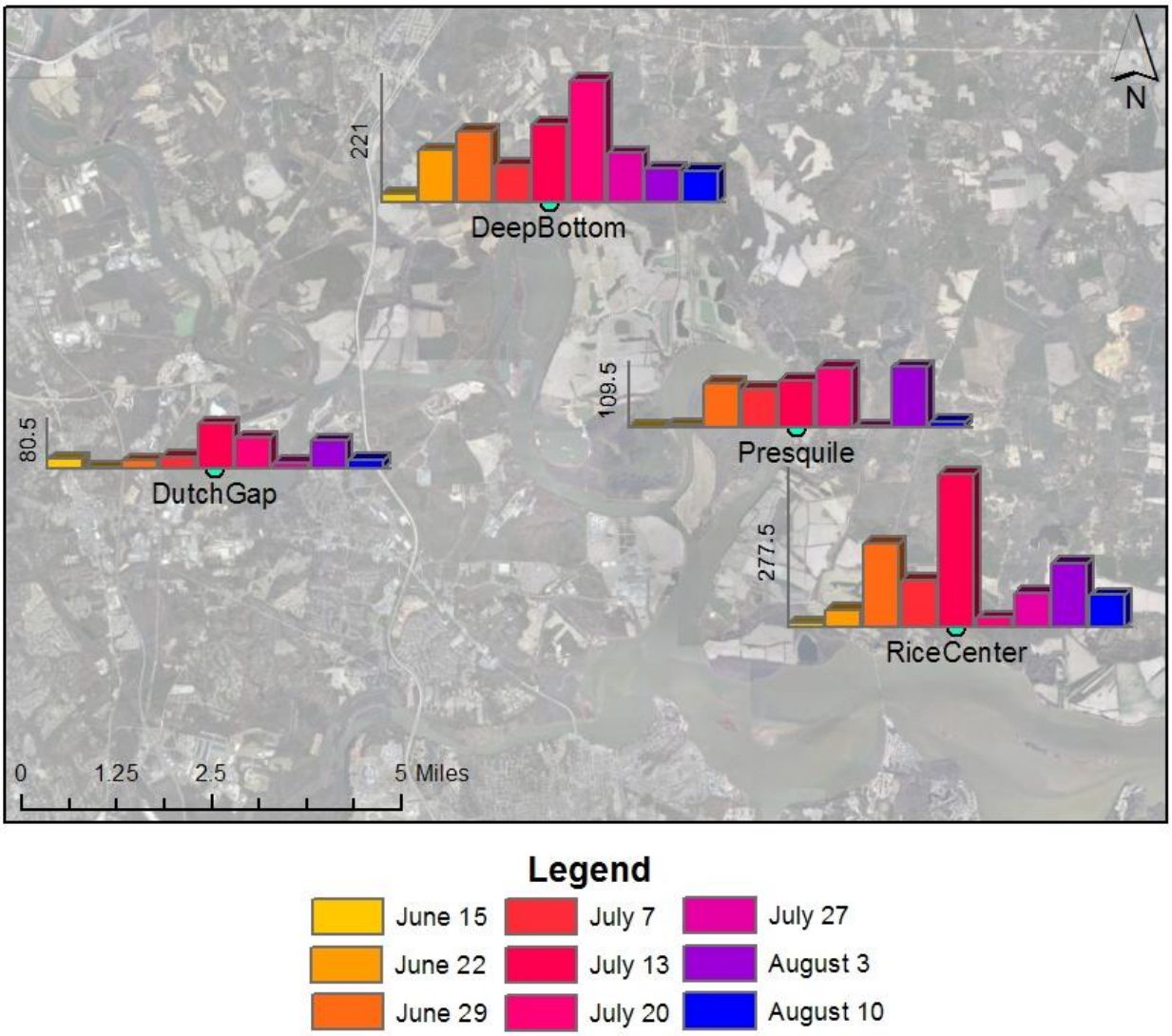

Figure 6. Distribution of *Culex erraticus* collected at each site per week

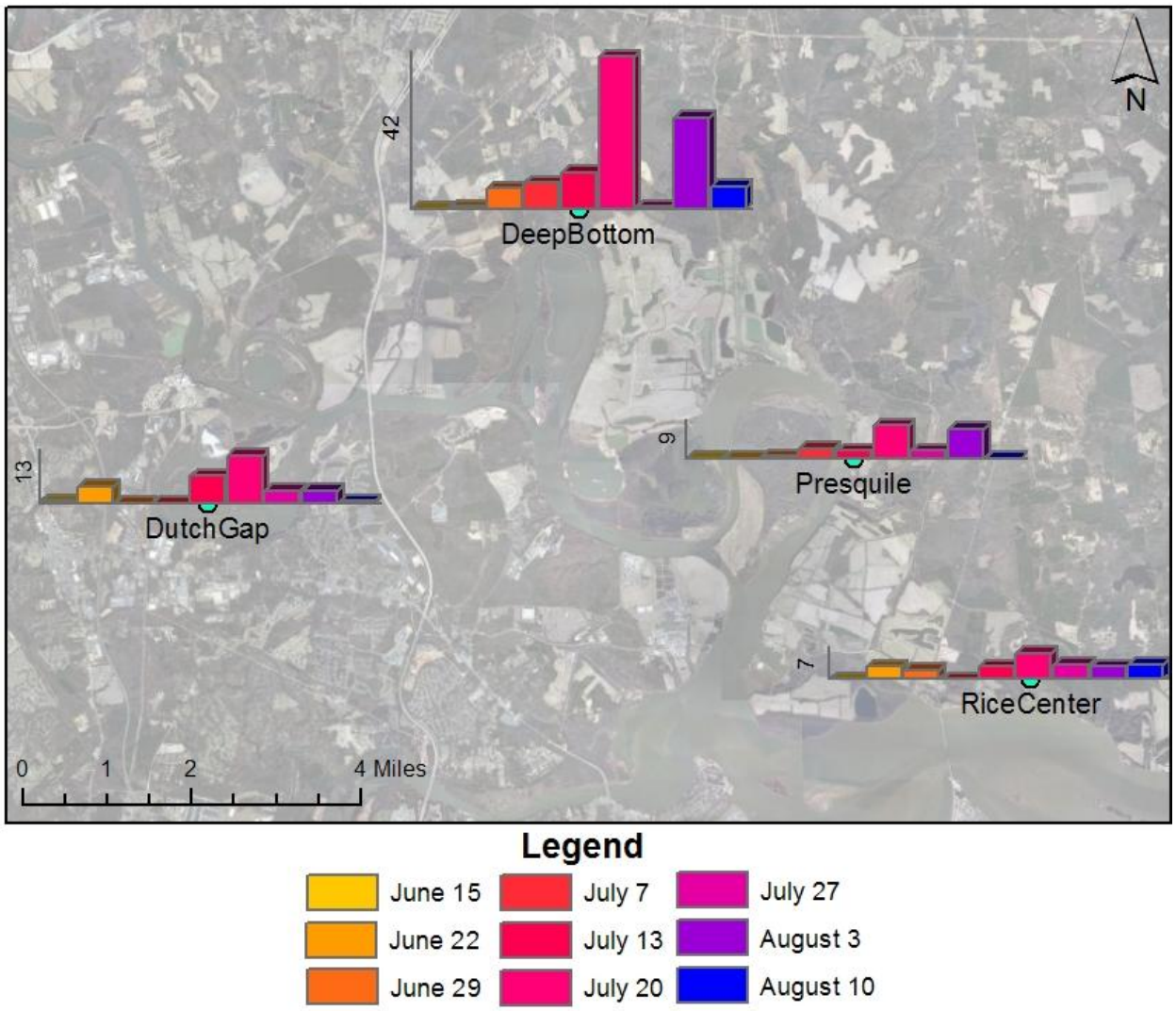

Figure 7. Distribution of *Culex pipiens/restuans* collected at each site per week

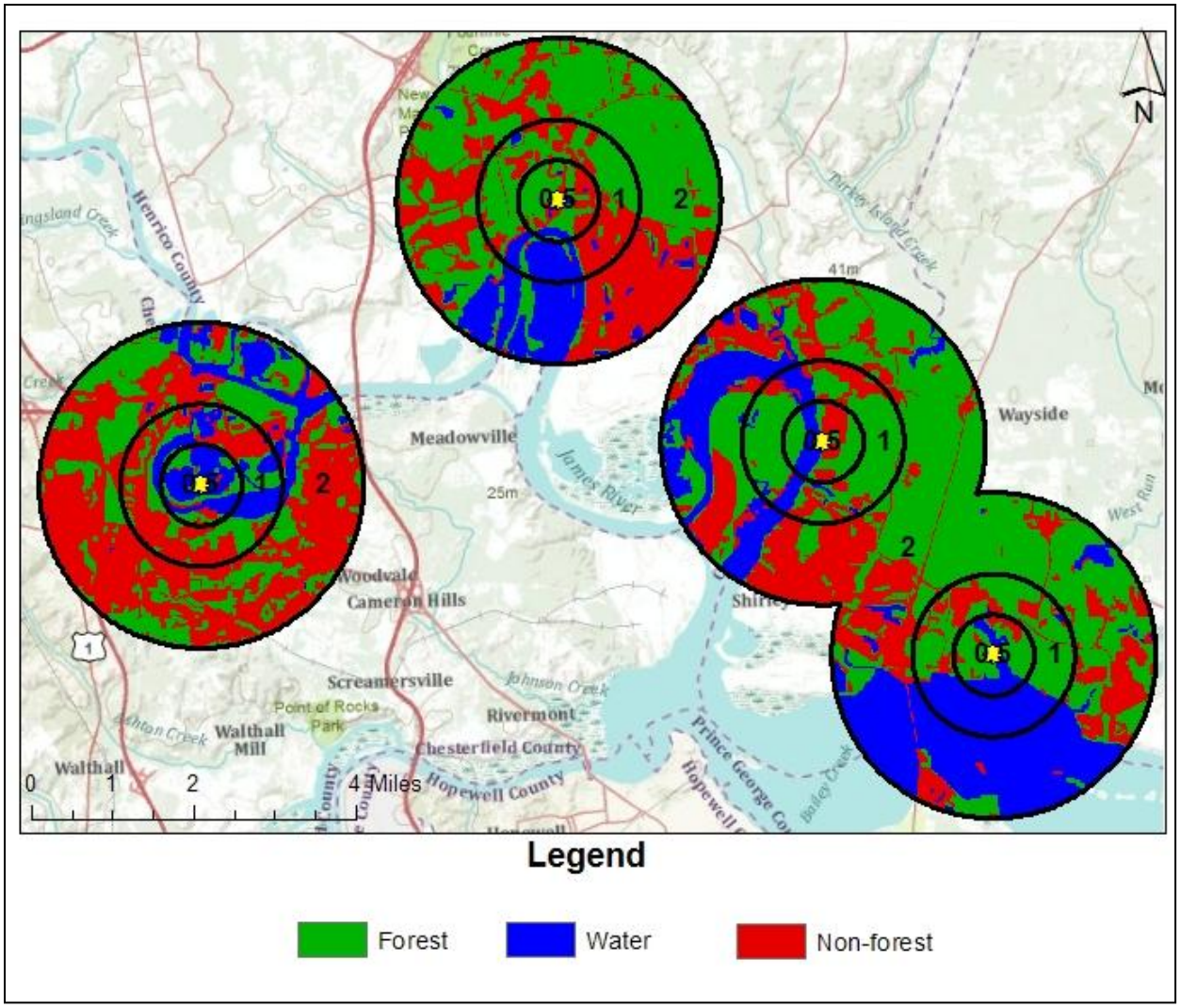

Figure 8. Map of forest cover data extracted within buffers surrounding each trap location

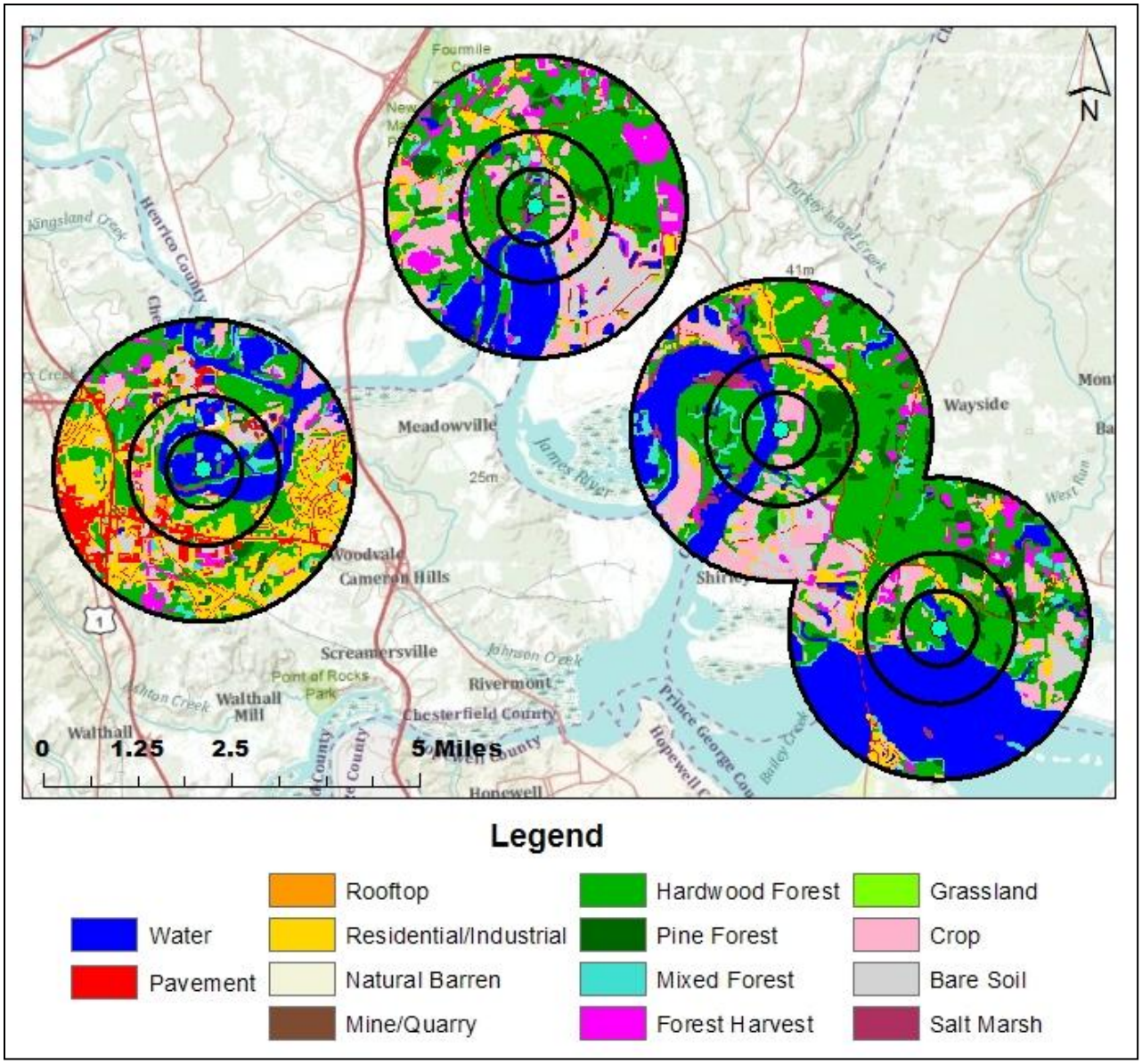

Figure 9. Map of land use data extracted within buffers surrounding each trap location

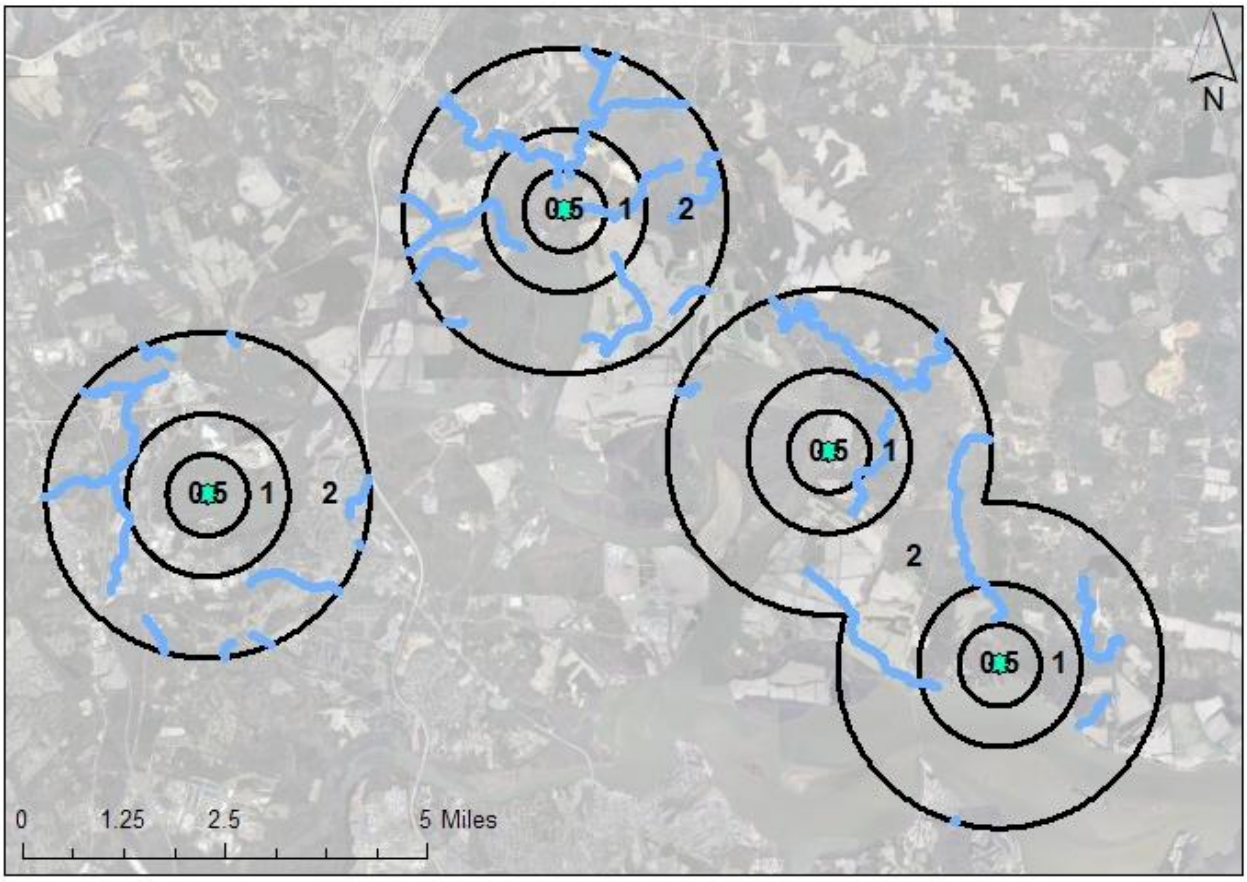

Figure 10. Map of impaired river data intersected within buffers surrounding each trap location

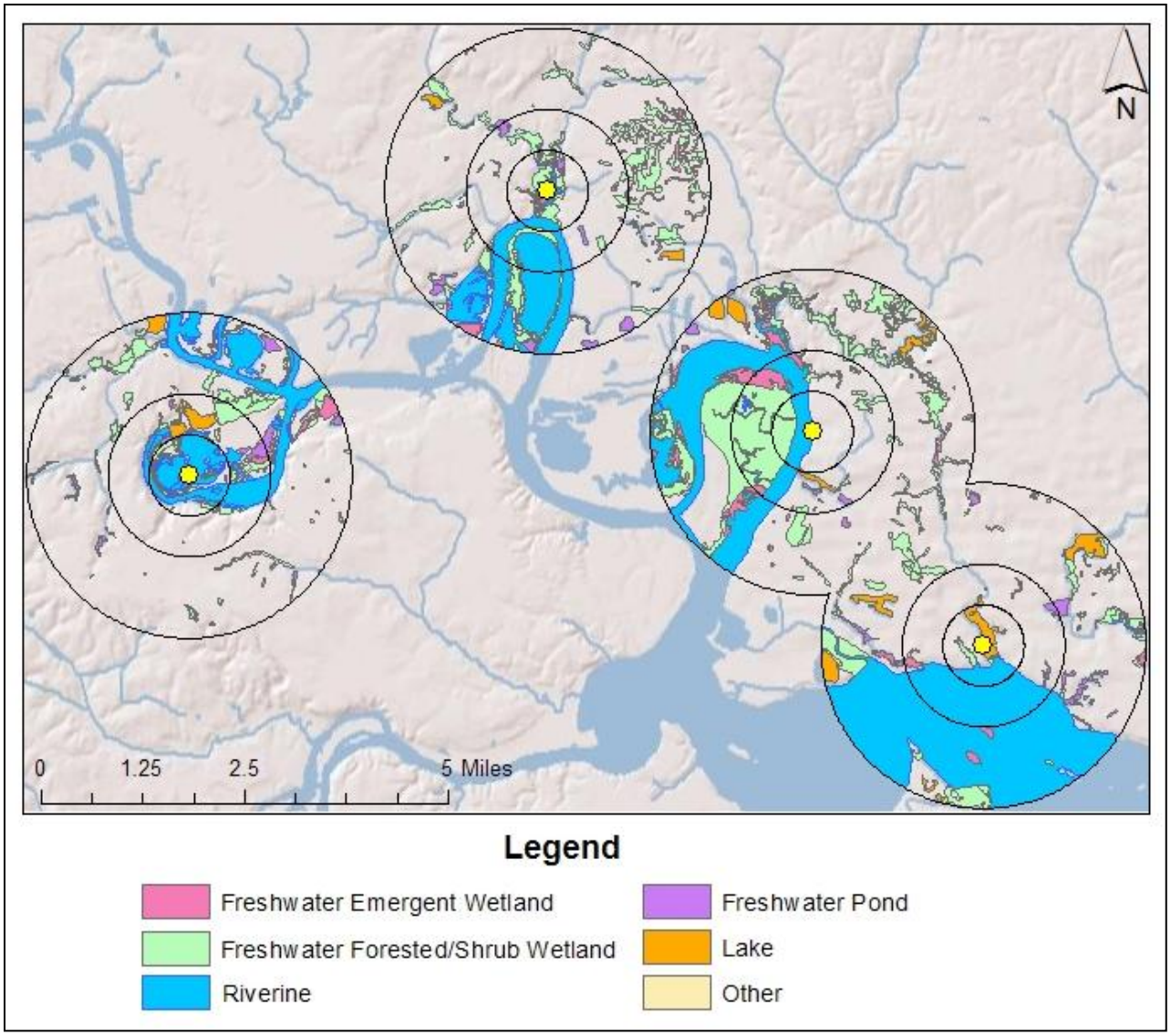

Figure 11. Map of wetland type data intersected within buffers surrounding each trap location

CHAPTER II: ANALYSIS OF BLOOD MEALS AND AVIAN MALARIA PARASITES OF MOSQUITO VECTORS COLLECTED ON PROTONOTARIA CITREA BREEDING GROUNDS IN CENTRAL VIRGINIA

INTRODUCTION

Avian Malaria and Transmission

The study of avian malaria and its transmission have become major areas of research and concern throughout the world. The vectors have become a focus of many studies in order to determine areas where diseases are being transmitted between individual birds. Not only is it important to know the identity and relative of vectors in an area, but it is important to understand the rates at which the parasite exist in vector populations (Ejiri *et al.*, 2008). Knowing this, it is possible to assess the risk and prevalence of the infection in an area or a specific population of organisms (Ejiri *et al.*, 2008). Because Prothonotary warblers are a migratory species, it is important to understand their ability to transmit the disease between locations. Brown *et al.* (2007) demonstrated a direct relationship between infected vectors of an area and the movements of migratory birds.

Mosquitoes as Vectors of Avian Malaria

Mosquitoes are known vectors for avian malaria. The parasites are transmitted to a mosquito from an infected host when the mosquito takes a blood meal. As the blood is processed by the female mosquito, the parasites, as gametocytes, develop into sporozoites and

migrate to the salivary gland over the course of 16 days (Ghosh *et al.*, 2003). From that point on, the mosquito is capable of infecting other hosts when it takes a blood meal. The level of infection a female mosquito is exposed to during blood meal consumption will affect the rate of infection and the amount of parasites that can be produced (Mahmood *et al.*, 2004).

Mosquito Blood Meal Identification

Mosquitoes can be either general or specific in their feeding patterns, and the host may vary between species. Many studies have been conducted with the objectives of analyzing the blood meal of mosquitoes in order to understand the disease ecology at the local level. Ngo *et al.* (2003) discussed determining the host of mosquito blood meals to understand the transmission of West Nile virus (WNV) and the spread of the disease by mosquitoes in an area. Kim *et al.* (2009) conducted a similar study in order to assess mosquito blood meals to determine the disease ecology of avian malaria. *Culex* species were targeted specifically in Louisiana by Mackay *et al.* (2010) due to their competency to transmit various diseases. Numerous other studies also seek to determine the host feeding patterns of specific species of mosquitoes (Kent *et al.*, 2009; Sawabe *et al*., 2010; Unlu *et al.*, 2010). Studies such as Hamer *et al.* (2009) also demonstrate the need to understand the host selection patterns of mosquitoes for disease transmission as they documented that *Culex pipiens/restuans* will switch feeding behavior between birds and humans. While such a feeding pattern does not pose a risk for humans in the case of avian malaria, the potential exists for mosquitoes to spread other infections between wildlife and humans.

Avian Malaria Lineages

Recent studies have also involved the assessment of various lineages of *Plasmodium*, attempting to determine transmission relationships between bird populations. Several

Plasmodium lineages have been identified in Hawaii, demonstrating that many lineages of one disease may exist in the same location (Beadell *et al.*, 2006). In the same study by Beadell *et al.* (2006), there was some indication that certain lineages of *Plasmodium* have become isolated in different locations in Hawaii. They also stated that these variations may be due to an unidentified incompatibility with vectors between different infected locations (Beadell *et al.*, 2006). Fallon *et al.* reported in a 2006 study that there was no evidence in the populations they tested that specific lineages of avian malaria were restricted to one breeding population, or even a geographical region. They determined that it would also be important to understand what occurred in terms of disease transmission at each point in the migratory route of a bird population (Fallon *et al.*, 2006).

Haemoproteus

Haemoproteus is a genus of parasites that belongs to the family Haemosporidia, and produces symptoms similar to malaria (Lainson, 1998). While parasites of this genus are not the same as those that cause avian malaria, the study of these parasites are important for understanding avian malaria due to their morphological, life cycle, and host similarities (Lainson, 1998). *Haemoproteus* may not affect an infected bird as negatively as avian malaria, but the similarities in hosts and transmission patterns are important areas of study (Bensch, 2000). *Haemoproteus* is known to infect a variety of hosts, from reptiles such as turtles to different species of birds (Garvin, 2003; Ishtiaq, 2008). The study of *Haemoproteus* provides important information with which to correlate *Plasmodium* information.

Role of Migratory Birds in Transmission

To understand the spread of avian malaria between bird populations, it is important to understand the role of migratory birds. Birds are known to be amplifying hosts for various

infections, giving parasites such as *Plasmodium* a location in which to multiply before dispersal (Lopez *et al.*, 2008). Because they migrate over a long distance, they may contract a disease and then carry it to new locations (Lopez *et al.*, 2008). Understanding the role of migratory birds in disease transmission is important for predicting the movement of infections to potentially vulnerable populations. Their interactions with species that are considered non-migratory are also important. Larger birds, such as corvids and owls, are known to transmit diseases, including those that can spread to humans (Gancz *et al.*, 2002; Fitzgerald *et al.*, 2003; Reisen *et al.*, 2006). The role of smaller bird species in the spread of disease is not as well understood, though some research has been done, as in the case of cliff swallows (Brown *et al.*, 2007). Thus, there is a need for evaluating how small birds, both migratory and non-migratory, spread disease in a location and between locations.

Objectives

Due to observed high rates of avian malaria infection in Prothonotary warblers at the four sites previously discussed, there is a need to assess the role of mosquitoes in those locations. The first objective of this chapter of the study is to determine the organisms on which the collected mosquitoes have fed. In doing so, the transmission of avian malaria can be better understood at the four investigated Virginia Prothonotary warbler breeding sites.

The second objective of this study was to determine the rate at which the mosquito populations of the area are infected with avian malaria. This will aid in determining if the parasite is spreading to other birds at the Virginia breeding grounds.

MATERIALS AND METHODS

Pooling

The mosquitoes used in this study were the same as those collected and described in Chapter 1. Blood fed mosquitoes were tested individually, for a total of 91 samples. All mosquitoes identified as not having recently imbibed a blood meal were pooled into groups of 10-50 by date of collection, trap type, location, and species. There were a total of 504 pools. Of those, 186 were randomly selected so that there was one pool for each date, trap type, location, and species. The heads were removed from all mosquitoes undergoing molecular testing.

DNA Extraction

Each of the 91 mosquitoes identified as blood-fed were tested individually. The head of each mosquito was removed to prevent interference with Polymerase Chain Reaction (PCR) (Kim *et al.*, 2009). Using the protocol of Kim *et al*. (2009), the mosquito abdomen was exposed and the DNA of the blood meal extracted using the REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich) with the following modification. The tissue extraction was performed at 55˚C.

As with the blood-fed samples, the non-blood-fed pools of mosquitoes were extracted using the REDExtract-N-Amp Tissue PCR Kit after removal of the heads (Kim *et al.*, 2009). Due to the high amount of tissue in some of the samples, reagents were doubled from the amounts specified by the kit. The first incubation temperature was also changed to 55˚C to insure complete extraction.

Identification of Blood Meal Host DNA by PCR

All of the following PCR reactions were done using the REDExtract-N-Amp Tissue PCR Kit (Kim *et* al., 2009). Each sample was tested using universal primers designed to amplify the cytochrome b sequence of DNA belonging to mammalian, avian, or amphibian hosts (Kim *et al.*,

2009). The primers used were VerU1 (5'-AAG ACG AGA AGA CCC TAT GGA-3') and VerU2 (5'-CCT GAT CCA ACA TAG AGG TCG TA-3'). The following program was used to amplify the universal DNA samples: 94˚C for 2 minutes, followed by 35 cycles of 94˚C for 30 seconds, 58˚C for 30 seconds, 72˚C for 90 seconds, and concluded with 72˚C for 4 minutes and a 4˚C holding temperature. Samples were electrophoresed on 1% agarose gel in TAE using a 100 bp ladder to target the expected 280 bp fragment. The gel was stained with 0.75 mg ethidium bromide and visualized with a UV light box. All subsequent gels were stained with the same procedure.

Avian Host Testing

Those samples from which DNA was successfully amplified using the universal primers were then tested for DNA from avian hosts. Using primers Avian-3 (5'-GAC TGT GAT AAA ATT CCA TTC CA-3') and Avian-8 (5'-GTC TTC ATT TTT TGG TTT ACA AGA C-3'), the products were amplified using the following program: 94˚C for 2 minutes, 35 cycles of 94˚C for 30 seconds, 55˚C for 30 seconds, 72˚C for 60 seconds, and concluded with an extension at 72˚C for 4 minutes and held at 4˚C. The samples were electrophoresed on a 1% agarose gel in TAE.

Mammalian Host Testing

Samples that did not test positive for avian DNA but did show amplification using the universal primers were tested for the presence of mammalian DNA. The primers Mammalian-1 (5'-TGA TAT GAA AAA TCA TCG TTG-3') and Mammalian-2 (5'-TGT AGT TAT CTG GGT CTC CTA-3') were used with the program settings 94˚C for 2 minutes, 35 cycles of 94˚C for 30 seconds, 55˚C for 30 seconds, and 72˚C for 90 seconds, with a final extension at 72˚C for 4 minutes and held at 4˚C. A 1% agarose gel in TAE stained with ethidium bromide was used to visualize the 772 bp fragment.

Amphibian/Reptilian Host Testing

To confirm the presence of amphibian DNA in those samples that tested positively for the universal primers but not the avian or mammalian hosts, primers designed to amplify a conserved cytochrome *b* sequence of amphibian and reptilian DNA were used. A positive control was provided by the lab of Dr. Amanda Dickenson of VCU. The primers Amphibian-1 (5'-CCC CTC AGA ATG ATA TTT GTC CTC A-3') and Amphbian-2 (5'-GCT GAT ACT TAT TTT GCT TTT TCT TC-3') were used in combination with the REDExtract-N-Amp Tissue PCR Kit instead of their original specified protocol (Cupp, 2004). The thermocycler settings for this reaction were as follows; 95˚C for 2 minutes, 55 cycles of 94˚C for 45 seconds, 50 seconds at 50˚C, and 1 minute at 72˚C, 1 minute at 72˚C, and the final extension was for 7 minutes at 72˚C. The samples were electrophoresed run on a 2% agarose gel. A positive control of amphibian DNA isolated by the lab of Dr. Amanda Dickenson was used.

Determining the Presence of Haemosporidia

Using illustra PuReTaq Ready-to-Go PCR beads (GE Healthcare Bio-Sciences Corp, Piscataway, NJ), the extracted mosquito samples were amplified using HaemF (5'-ATG GTG CTT TCG ATA TAT GCA TG-3') and HaemR2 (5'-GCA TTA TCT GGA TGT GAT AAT GGT-3') primers (Waldenstrom *et al*., 2004). The thermocycler was programmed to 94˚C for 3 minutes, 35 cycles of 94˚C for 30 seconds, 50˚C for 30 seconds, 72˚C for 45 seconds, and a final extension step of 72˚C for 10 minutes with the final hold at 4˚C. After the PCR reaction was complete, the samples were electrophoresed on 1.2% agarose gels and stained with ethidium bromide, as were all subsequent gels. A positive control was provided by a Haemosporidia positive sample from the study of Elena Grillo.

Determining the Presence of *Plasmodium*

Those samples that tested positive for the presence of Haemosporidia were then tested for the presence of *Plasmodium*. Illustra PuReTaq Ready-to-Go PCR beads (GE Healthcare Bio-Sciences Corp, Piscataway, NJ) were used to amplify the target 524 bp fragment using primers FP3 (5'-TAT ATA ACT TTT TTG ATA TG-3') and RP3(5'-GTT ATT GCA TTA TCT GGA TGT GA-3') (Waldenstrom *et al*., 2004). The following program was used to complete the PCR reaction: an initial 94˚C for 1 minute, followed by 40 cycles of 94˚C for 20 seconds, gradient 52.9˚C for 20 seconds, and 72˚C for 30 seconds, concluded with an extension at 72˚C for 10 minutes and the samples were held at 4˚ C. The samples were electrophoresed on a 1.2% agarose gel. A *Plasmodium* positive bird blood sample isolated by Elena Grillo was used for the positive control.

Determining the Presence of *Haemoproteus*

The samples that tested positive using Haemosporidia primers were also tested for *Haemoproteus*. Primers FH3 (5'-GAT TRA ACT CAT TTT TTG TTT TTA CT-3') and RH3 (5'-ACA ATT GCA TTA TCA GGA TGA GC-3') were used to amplify the target 524 bp (Waldenstrom *et al*., 2004). The illustra PuReTaq Ready-to-Go PCR beads were used with the program of 94˚C for 1 minute, 40 cycles of 94˚C for 20 seconds, gradient 52˚C for 20 seconds, and 72˚C for 30 seconds, followed by a final extension at 72˚C for 10 minutes and held at 4˚C. The final products were electrophoresed on a 1.2% agarose gel. The positive control was a *Haemoproteus* positive bird blood sample from the study of Elena Grillo.

Statistical Analysis

Fisher's exact test was used to determine if there were significant differences between the mosquito species based on host DNA extracted from the blood fed mosquitoes. This was

performed in R 2.7.1 using α=0.05. The test was also used to determine if there was a significant difference between sites based on infection rate. Due to the pooling method, the non-blood fed samples could not be statistically analyzed.

RESULTS

Host DNA Analysis

Of the 91 tested samples, 83 (91.2%) showed positive amplification with the universal primers used indicating that the collected mosquitoes have fed on either amphibian, mammalian, or avian hosts, while 8 did not. An example gel is found in Figure 12. Of those 83, 48 (57.8%) positively amplified in the presence of amphibian/reptilian primers (Figure 13), 7 (8.4%) resulted in positive amplification with the avian primers (Figure 14), and 1(1.2%) sample showed positive amplification with mammalian primers (Figure11) (Table 11). The positive samples were from *Cx, erraticus, Cx. salinarius, Aedes vexans Coquillettidia perturbans, Uranotaenia sapphirina,* and *Anopheles quadrimaculatus* (Table11). There was a significant difference between the mosquito species based on the universal primer amplification results, (p=0.014), but not between the host specific primers.

Haemosporidia Prevalence

Among the 91 blood fed samples, 72 (79.2 %) tested positively for the presence of Haemosporidia (Figure 16; Table 12). Of the blood fed mosquitoes that tested positive for the presence of host DNA using universal primers, 19 (22.9%) of those tested positively for Haemosporidia (Table 12). There was a significant difference between the species of mosquito that amplified for Haemosporidia (p=0.02) Of the non-blood fed mosquito samples, most of the positive results came from Deep Bottom, with a total of 31(16.7%) out of the 186 samples tested

resulting in positive amplification for Haemosporidia (Table 12). *Culex erraticus* was the predominant mosquito species to test positive for infection with Haemosporidia (Table 13).

Plasmodium Prevalence

Of the 91 blood fed mosquito samples, 10 (11%) tested positive for the presence of *Plasmodium* (Table 12). There were 4 species of mosquito that tested positive, with *Cx. erraticus* being the most common (Table 12). There were 14 positive samples among the 189 non-blood fed samples tested, with most of the samples originating from Deep Bottom (Figure 17; Table 13). *Cx. erraticus* was the most prevalent species that tested positive for *Plasmodium* (Table 13).

Haemoproteus Prevalence

Fifteen (7.9%) of the 189 samples tested were positive for *Haemoproteus* infection (Figure 18; Table 13). Of those, the species distribution varied by site (Table 13). At Presquile, *An. crucians* was the species that had the most positives; at Dutch Gap *Cx. erraticus* was the species that resulted in the most positives, and *Cx. salinarius* was the highest species with positive amplification at Deep Bottom and the Rice Center. Among the 91 blood fed samples, 10 (11%) tested positive for *Haemoproteus*, with the most positives carried by *Cx. erraticus* (Table 11-12). This infection was carried by *Cx. erraticus* in nine samples, with one *An. quadrimaculatus* being positive for *Haemoproteus* (Table 12).

DISCUSSION

Of the 91 blood fed mosquitoes tested, 83 (91.2%) tested positively with the universal primers. This demonstrates a high level of successful host DNA extraction. Further analysis determined that 7 were from avian hosts, 1 was from a mammalian host, and 48 were from

amphibian or reptilian hosts. This demonstrates a wide range of hosts that are providing the female mosquitoes with blood meals.

Even though only seven mosquitoes fed on avian hosts (7.7%), the results are still indicative of mosquitoes biting birds at the Prothonotary warbler breeding sites. Of those, 3 were *Cx. erraticus*, while 4 were *Cx. salinarius*. This is important because while *Cx. pipiens* is a known bird biting mosquito, these two species are more opportunistic and will feed on large mammals as well as birds (Fairfax County Health Department; Mackay, 2010; Sawabe, 2010). Thus, these species are potentially acting as vectors for avian malaria in these areas. They may also have the capability of spreading disease from birds to mammals, including humans. However, due to the low number of mammalian positive samples (1.1%), it appears that there are few mosquitoes biting mammals. According to these results, there are mosquito species, notably *Cx. erraticus*, on the Prothonotary warbler breeding grounds that are feeding on more than one type of host, showing a more opportunistic feeding pattern.

Further, 52.7% of the mosquito blood meals tested positive for amphibian and reptilian hosts. Among those positives, there were five samples that had not previously amplified using the universal primers. This provided evidence that the universal primers, as they were designed, did not result in positive amplification in the presence of reptilian blood. The five samples that tested positive in the presence of the amphibian/reptilian primers but not in the presence of the universal primers provide evidence of the mosquitoes biting reptiles. Comparatively the mosquitoes sampled at the *P. citrea* breeding sites seem to bite amphibians or reptiles more often than birds and mammals. This could mean that while mosquitoes at the Prothonotary warbler breeding sites are biting birds and mammals, amphibians or reptiles may provide greater feeding opportunities. This would mean that the level of transmission of any avian diseases would not be

as high as anticipated and there could be other factors influencing avian malaria transmission. Considering the known prevalence of avian malaria at those sites, these results were unexpected. There has been evidence that suggests that corvids can transmit WNV even in the absence of mosquitoes, and that passerines expel a high amount of WNV through the cloaca (Komar *et al.*, 2003). It has also been found that females among blue tits in England had a higher amount of infection of avian malaria (Wood *et al.*, 2007). Given this, female nest sitting combined with direct contact to nestlings could be transmitting the infection, partially explaining the high infection rate among the collected mosquitoes.

In addition to *Cx. salinarius* and *Cx. erraticus, Co. perturbans, Ur. sapphirina, Ae vexans,* and *An. quadrimaculatus* also demonstrated positive amplification of host DNA using universal primers (Table 11). *Ae. vexans* and *Ur. sapphirina* were found to have fed on amphibian or reptilian hosts. *An. quadrimaculatus* amplified positively in the presence of the universal primers, but not in any of the subsequent primer analyses. This may have been due to non-specific binding or degraded DNA. The results demonstrated that there are numerous species at the Prothonotary warbler breeding grounds that can be successfully examined for blood meal information, and that specific mosquito species cannot be discounted in blood meal analysis. At Dutch Gap, Deep Bottom, and Presquile, all positively identified blood meals were from amphibian or reptilian hosts (Table 11). All avian and mammalian positive samples were from the Rice Center, and there were still a high number of samples that resulted in positive amplification using amphibian/reptilian primers (Table 11). This is potentially problematic, as this means there is no way to determine from these mosquitoes if the mosquitoes at Dutch Gap, Deep Bottom, or Presquile are biting birds. This brings into question the mosquitoes acting as effective avian malaria vectors at these locations. Due to this, more accurate mosquito blood

meal studies are needed. Such studies would include more accurate methods of collecting blood fed mosquitoes and take relative abundances of available host species in order to determine the feeding preferences of mosquito species. More appropriate mosquito trap placement, such as closer to the bird boxes, is also something to consider in the future. Of those mosquitoes collected, 91 were blood fed. This is a relatively low number in comparison to the total number of mosquitoes collected. Due to this, it would be beneficial in the future to change the sampling methods in order to capture more blood fed mosquitoes. This may include the use of resting boxes or aspiration as these methods are more likely to catch blood fed mosquitoes.

It is important to note that there were several samples that originally amplified positively using the universal primers, but did not demonstrate any positive amplification at the host level. The reason for this has not been determined, but it is thought to be caused by non-specific primer binding in the universal primer analysis or degraded DNA.

There is a need to determine the exact host species on which the mosquitoes are feeding. In future studies, it would be beneficial to sequence the PCR product to determine the host. This would be especially important for determining if any of the avian samples were actually a Prothonotary warbler. This would be especially important for those locations that did not have avian positive samples. There may be a mix of DNA that was not successfully or completely detected by PCR, as mosquitoes are known to take multiple blood meals. Another caution is that the prevalence of avian malaria at the Rice Center is currently unknown, and this will need to be determined in the future.

Parasite Analysis

Among those tested, 79.1% of the blood fed mosquitoes were found to be positive for Haemosporidia infection providing evidence that the mosquitoes collected from the four

breeding sites of Prothonotary warblers carried the parasites responsible for the infection in the birds. However, only 31 of the 186 pools of non-blood fed mosquitoes tested positively for the presence of Haemosporidia (Table 13). This data then indicates that there are not many mosquitoes at the Prothonotary warbler breeding sites that are in contact with and capable of spreading the parasites. This is also true for *Plasmodium* and *Haemoproteus.* There were even fewer positive samples among the non-blood fed samples that tested positively for the individual genera (Table 14). Among the positive samples, Deep Bottom was the location with the highest prevalence (Table 13). It was also determined that there was a significant difference between the mosquito species that tested positive for Haemosporidia (p=0.021) and *Haemoproteus* (p=0.012), but not *Plasmodium* among the blood fed samples. Thus there are mosquito species which are important vectors for *Haemoproteus*, but not *Plasmodium*. Among the non-blood fed samples, there were no significant differences between the sites according to the level of parasite infection. This means that further analyses are needed to accurately determine which species of mosquitoes are the acting as vectors for *Plasmodium.*

At all locations, *Cx. erraticus* was the primary species that was found to carry the parasites. This was true for both blood fed and non-blood fed mosquitoes. While this species is considered more opportunistic than *Cx. pipiens*, a known bird biting mosquito, it may feed on birds as well as other hosts (Fairfax County Health Department). Table 14 demonstrates the differences in species that tested positive during analysis of the non-blood fed samples. The majority came from *Culex* species, which was expected, while there were also a number of positives from *Anopheles* species. Both genera demonstrate the possibility of not only transmission of infection between birds, but between larger mammals. This is important when

considering other infections such as WNV, which can be transmitted to humans from birds via mosquitoes.

Finally, my methods allowed it to be established that the mosquitoes were infected, but not necessarily infectious. The bodies of the mosquitoes were not dissected past removing the heads. This means that further analysis would have to be done to conclude whether the mosquitoes are capable of transmitting the parasites. For the non-blood fed samples, the mosquito had the parasite somewhere in its body, but it was not possible to determine if the parasites had moved to the salivary gland for future transmission or if were still only present in the abdomen. For the blood fed samples, this is further compounded by the fact that the parasite DNA may exist in the blood meal and not in the mosquito itself. In the future, the mosquitoes could be dissected in such a way to answer these questions. The salivary gland could be removed to determine the infectivity of the mosquito, while the abdomen would be used for blood meal analysis (Mourya, 2001). From the current analysis, it is known that some of the mosquitoes at the four Prothonotary warbler breeding sites were positive for *Plasmodium* and *Haemoproteus* species DNA, but the species of mosquitoes acting as vectors is still unclear.

Parasite and Blood Meal Analysis

The blood meal analysis combined with the parasite analysis yielded unexpected results. First, there were very few mosquitoes that had fed on birds. However, a number of the mosquito samples, both blood fed and non-blood fed, tested positive for the presence of parasite DNA (Tables 13 and 14). Of the seven samples that were identified to have avian blood, only three tested positive of Haemosporidia and two of those for *Plasmodium* (Table 11-13). The mammalian positive samples and several of the amphibian/reptilian samples also tested positive for the presence of parasite DNA (Tables 11-13).

All of the avian blood samples came from the Rice Center, but there were mosquitoes from all locations infected with parasite DNA (Table 12). This demonstrates that the mosquitoes are taking more than one blood meal over the course of a season from different hosts. While they have avian parasites in their system, the blood meal at the time of collection was not from an avian host. This in turn demonstrates that it is possible for mosquitoes to become infected with an avian parasite and potentially pass it on to another host when it takes another blood meal.

Future Directions

In the future, several questions should be addressed. The first is which species are acting as vectors for *Plasmodium* and *Haemoproteus*. This study indicates that they are capable of doing so and will bite avian hosts, but the species of mosquitoes acting as vectors is still unclear. This in turn indicates a need to determine if there are other factors influencing transmission. In conclusion, the mosquitoes at the Prothonotary warbler breeding sites are infected with the causative agents of avian malaria.

Sampling methods may also be improved to see if more mosquitoes are carrying or transmitting the avian parasites. Traps set more closely to Prothonotary warbler next boxes may increase the rate of those mosquitoes that are carrying avian parasites and those that are biting the Prothonotary warblers. This will determine the mosquito species involved in the transmission of avian malaria in the Prothonotary warblers breeding sites in central Virginia.

Table 11. Host identification of mosquito blood meals from *P. citrea* breeding sites

Genus	Species	Number Tested	Blood Meal Results							
			Universals	Avian	Avian Proportion	Mammalian	Mammalian Proportion	Amphibian/ Reptilian	Amphibian/ Reptilian Proportion	Other/ Error
Aedes	*albopictus*	3	3	0	0.0%	0	0.0%	0	0.0%	100.0%
Aedes	*vexans*	7	4	0	0.0%	0	0.0%	4	100.0%	0.0%
Anopheles	*quadrimaculatus*	2	2	0	0.0%	0	0.0%	0	0.0%	100.0%
Coquillettidia	*perturbans*	2	2	0	0.0%	0	0.0%	2	100.0%	0.0%
Culex	*erraticus*	34	31	3	9.7%	1	3.2%	21	67.7%	19.4%
Culex	*pipiens/restuans*	9	8	0	0.0%	0	0.0%	4	50.0%	50.0%
Culex	*salinarius*	30	30	4	13.3%	0	0.0%	16	53.3%	33.3%
Psorophora	*ferox*	1	0	0		0		0		
Uranotaenia	*sapphirina*	3	3	0	0.0%	0	0.0%	2	66.7%	33.3%

Table 12. Infection status of mosquitoes collected on *P. citrea* breeding sites

Genus	Species	Number Tested	Infection Status Results					
			Haemosporidia	Haemosporidia Proportion	*Plasmodium*	*Plasmodium* Proportion	*Haemoproteus*	*Haemoproteus* Proportion
Aedes	*albopictus*	3	0	0.0%	0	0.0%	0	0.0%
Aedes	*vexans*	7	2	28.6%	1	50.0%	0	0.0%
Anopheles	*quadrimaculatus*	2	2	100.0%	1	50.0%	1	50.0%
Coquillettidia	*perturbans*	2	0	0.0%	0	0.0%	0	0.0%
Culex	*erraticus*	34	12	35.3%	7	58.3%	9	75.0%
Culex	*pipiens/restuans*	9	0	0.0%	0	0.0%	0	0.0%
Culex	*salinarius*	30	3	10.0%	2	66.7%	0	0.0%
Psorophora	*ferox*	1	0	0.0%	0	0.0%	0	0.0%
Uranotaenia	*sapphirina*	3	0	0.0%	0	0.0%	0	0.0%

Table 13. Haemosporidia infection status of non-blood fed mosquitoes collected from *P. citrea* breeding sites

	Positives by Location		
Location	Haemosporidia	*Haemoproteus*	*Plasmodium*
Dutch Gap	7	2	3
Rice Center	7	6	5
Presquile	4	1	0
Deep Bottom	13	6	6

Table 14. Distribution of Haemosporidia infection among species of mosquitoes collected on *P. citrea* breeding sites

Positives by Location and Species				
Location	Species	Haemosporidia	Haemoproteus	Plasmodium
Dutch Gap				
	Culex erraticus	3	1	1
	Culex salinarius	4	0	1
	Anopheles punctipennis	1	1	1
	Anopheles quadrimaculatus	1	1	1
Rice Center				
	Culex erraticus	2	2	1
	Culex pipiens/restuans	1	1	1
	Culex salinarius	2	1	1
	Anopheles crucians	4	4	4
	Anopheles punctipennis	2	2	2
	Anopheles quadrimaculatus	1	1	1
Presquile				
	Culex erraticus	4	1	0
	Culex pipiens/restuans	1	0	0
	Culex salinarius	1	1	0
Deep Bottom				
	Culex erraticus	5	4	2
	Culex pipiens/restuans	4	1	2
	Culex salinarius	3	2	1
	Culex unknown	1	1	1
	Anopheles crucians	6	2	4
	Anopheles punctipennis	6	2	4
	Anopheles quadrimaculatus	6	2	4

FIGURES

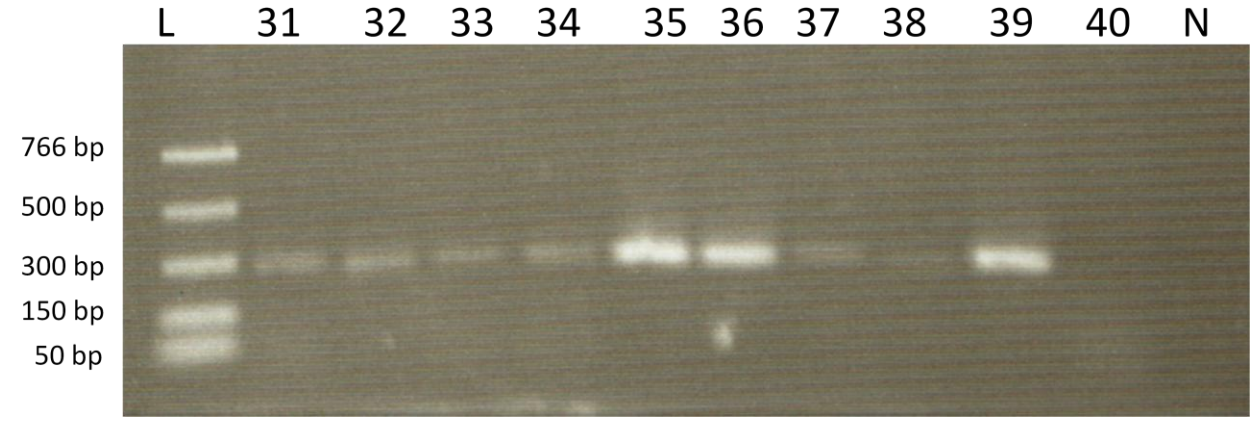

Figure 12. Gel electropohoresis image of mosquito blood meal amplified with universal primers

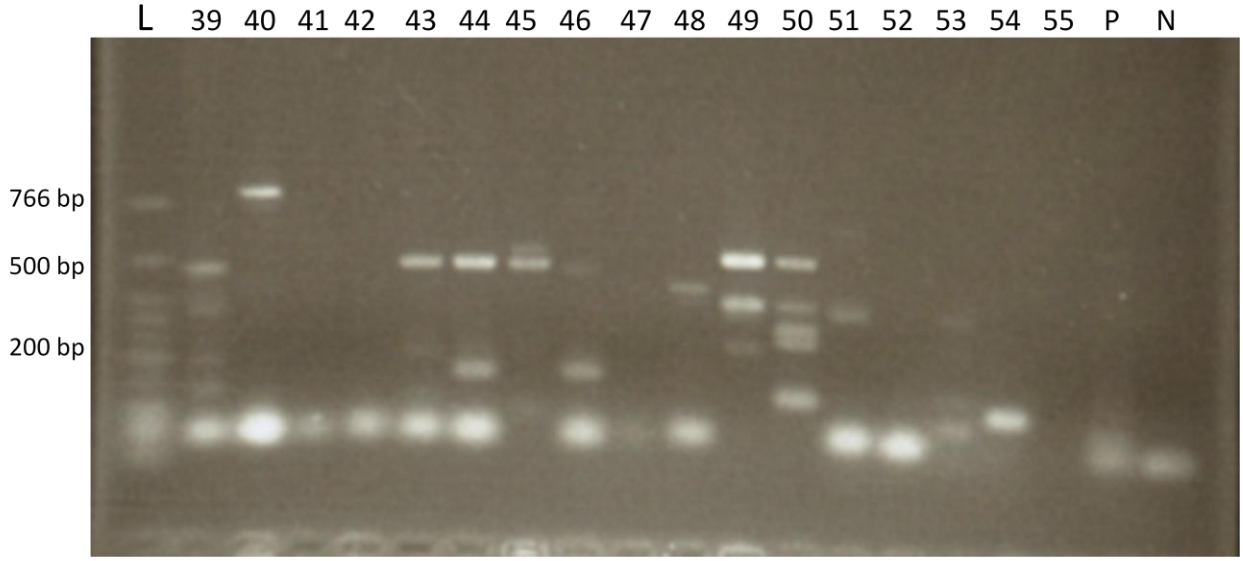

Figure 13. Gel electropohoresis image of mosquito blood meal amplified with amphibian/reptilian primers

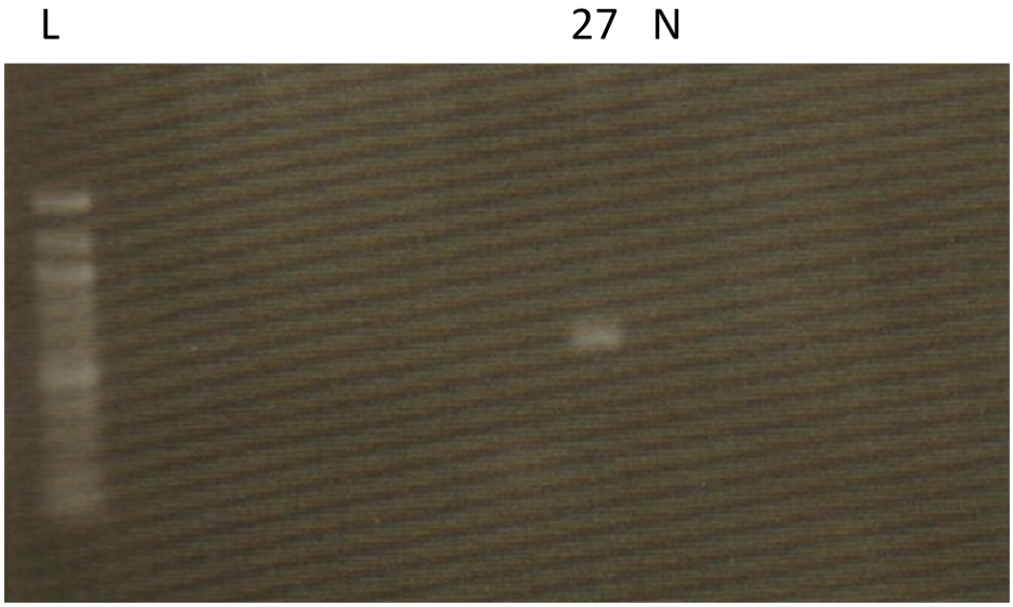

Figure 14. Gel electropohoresis image of mosquito blood meal amplified with avian primers

Figure 15. Gel electropohoresis image of mosquito blood meal amplified with mammalian primers

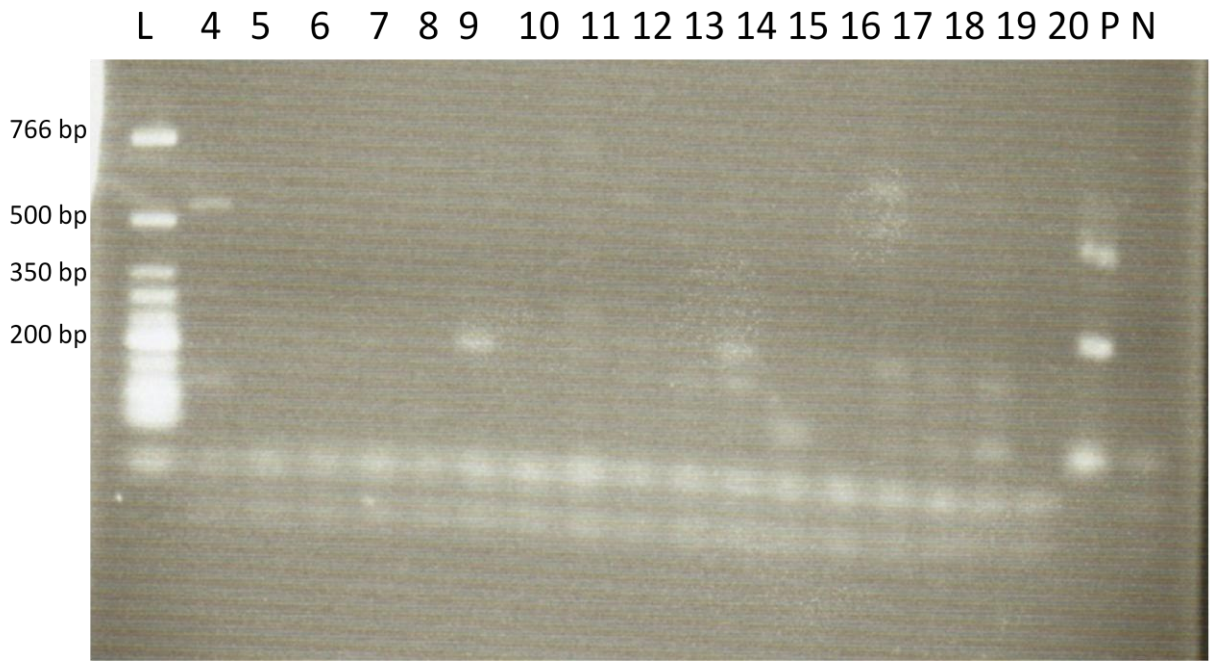

Figure 16. Gel electropohoresis image of mosquito blood meal amplified with Haemosporidia primers

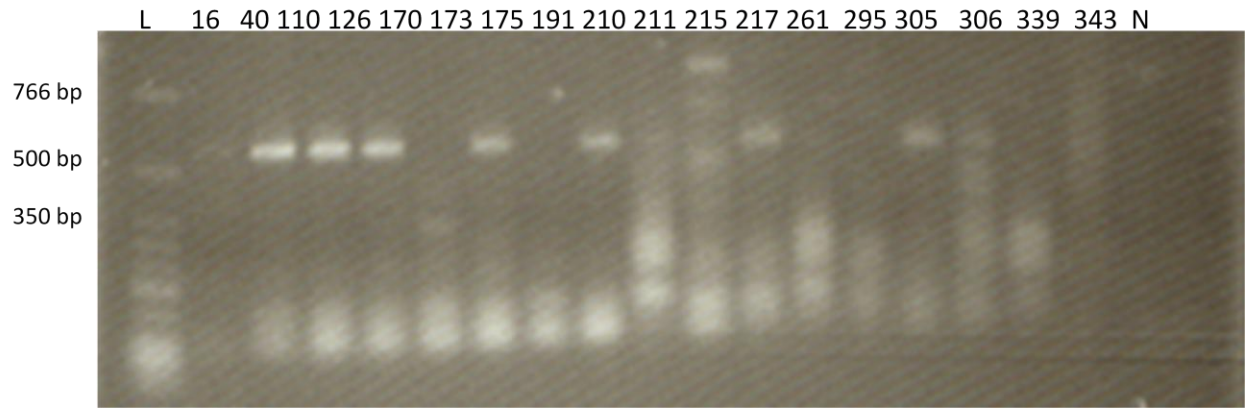

Figure 17. Gel electropohoresis image of mosquito blood meal amplified with *Plasmodium* primers

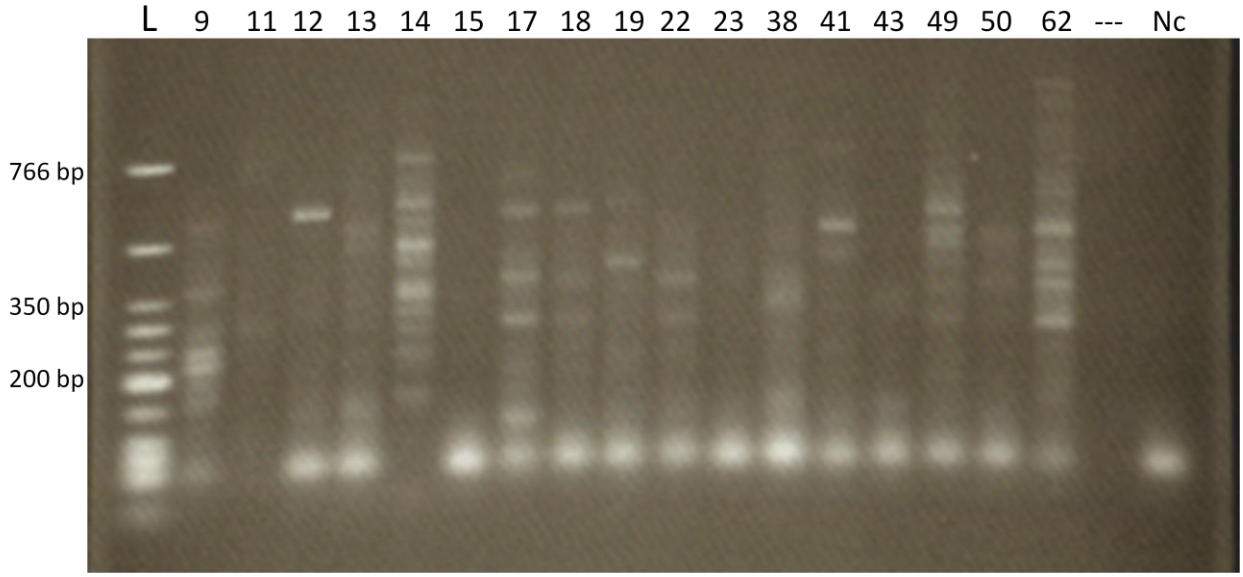

Figure 18. Gel electropohoresis image of mosquito blood meal amplified with *Haemoproteus* primers

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VITA

Catherine Marie Wallace was born in Virginia Beach, Virginia, United States on September 23, 1986. She was raised there and attended Floyd E. Kellam High School where she graduated in the spring of 2004 to attend Randolph-Macon College in Ashland, Virginia in the fall of 2004. Attendance was made possible by the Randolph-Macon Presidential Scholarship, which she maintained for all four years. While there, she was a departmental assistant for the biology department and volunteered at a veterinary hospital. In 2006 she received the Stephen H. Watts Award in Biology from the college. In the summers of 2006 and 2007, she completed two research projects under the Summer Undergraduate Research Project (S.U.R.F.), exploring the life of parasitic fungi. The first of her projects was presented at the 18th Annual Saint Joseph's University Sigma Xi Student Research Symposium in the fall of 2007. She was offered and accepted membership to several societies, which were the Beta Beta Beta Biological Honor Society, the Sigma Eta Phi Classical Honor Society, and The Society of Success and Leadership. Her Bachelor's degree in Biology and Classics with a minor in Chemistry was completed in the spring of 2008. Following this, she attended Virginia Commonwealth University to complete her Master's degree. She received a Graduate Teaching Assistantship, and taught a laboratory

methods class for four semesters. While there, she also received Walter and Inger Rice Center for Environmental Studies grants in the springs of 2009 and 2010. In her final semester, she was supported by an assistantship from the Graduate School at Virginia Commonwealth University which she had been awarded. In addition to her own thesis, she contributed to a manuscript titled *The Relationship between Haemosporidia infection and Ultraviolet Breast Feather Reflectance in Prothonotary Warblers (Protonotaria citrea)* submitted to *The Auk*. Catherine graduated from Virginia Commonwealth University in the fall of 2010 with a Master's degree in biology.